

Table 2.

Estimations of quantity, sugar- and nitrogencontent of urine and of the bloodsugar after loading in rats; alloxan was given before adrenalectomy.

	Body weight	Excretion in successive periods of 3 days each of					Bloodsugar	
		urine in ml.	sugar in gm.	nitrogen in gm.			Before loading	After loading
6 Normal rats	1st day 178	1st per. 21	1st per. 0	1st per.	0.57			
	5th day 171	2nd per. 19	2nd per. 0	2nd per.	0.56			
	8th day 168	3rd per. 21	3rd per. 0	3rd per.	0.53			
	11th day 169	4th per. 24	4th per. 0	4th per.	0.55	110	170	
	14th day 172	5th per. 27	5th per. 0	5th per.	0.57	(90—120)	(145—200)	
9 Adrenal-ectomized rats	1st day 195	1st per. 21	1st per. 0	1st per.	0.54			
	5th day 188	2nd per. 18	2nd per. 0	2nd per.	0.55			
	8th day 178	3rd per. 14	3rd per. 0	3rd per.	0.52			
	11th day 183	4th per. 17	4th per. 0	4th per.	0.55	90	130	
	14th day 185	5th per. 17	5th per. 0	5th per.	0.56	(60—110)	(110—150)	
10 Normal alloxan rats	1st day 204	1st per. 70	1st per. 5.4	1st per.	0.82			
	5th day 183	2nd per. 57	2nd per. 4.2	2nd per.	0.77			
	8th day 176	3rd per. 49	3rd per. 2.5	3rd per.	0.73			
	11th day 167	4th per. 46	4th per. 2.5	4th per.	0.75	225	435	
	14th day 168	5th per. 42	5th per. 1.7	5th per.	0.69	(105—330)	(325—600)	
9 Adrenal-ectomized alloxan rats	1st day 185	1st per. 55	1st per. 3.5	1st per.	0.65			
	5th day 177	2nd per. 35	2nd per. 0.7	2nd per.	0.60			
	8th day 180	3rd per. 25	3rd per. 0.4	3rd per.	0.58			
	11th day 181	4th per. 26	4th per. 0.2	4th per.	0.56	75	290	
	14th day 182	5th per. 25	5th per. 0.1	5th per.	0.58	(65—110)	(125—445)	

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CRYSTALLINE HUMAN CHORIONIC GONADOTROPHIN AND ITS BIOLOGICAL ACTION

By

L. CLAEßON, B. HÖGBERG, TH. ROSENBERG AND A. WESTMAN

A vast amount of literature has accumulated on the relationship between the hypophyseal-diencephalic system and the gonads. However, all the investigations for the elucidation of this important question were carried out with indirect methods, as a pure gonadotrophin preparation with uniform biological effect was not available. The same conditions characterized also the treatment of the diseases due to the disorders of the above mentioned relationship, manifested by pathologically changed sexual function.

A solution of these problems would be greatly facilitated by the use of chemically pure gonadotrophin hormones with well defined pharmacodynamical properties. Using these pure gonadotrophins remedies would be given to the gynecologists to treat diseases of the type mentioned above with a well founded and adequate therapy. Furthermore, such treatment may result in a better understanding of the action of the gonadotrophic hormones on the human organism.

In this paper we will describe a method for the preparation

of crystalline, electrophoretically homogeneous chorionic gonadotrophin. Preliminary results concerning the action of this new preparation on both laboratory animals and human beings are also presented.

EXPERIMENTAL

Chemical Investigations.

Since the classical investigations of *Ascheim* and *Zondek* (1927) have shown that the urine excreted during pregnancy in the human subjects contains great amounts of gonadotrophic hormone, numerous experiments have been carried out to obtain this hormone from the urine in a chemically pure form (*Chow*, 1944.)

Then *Gurin*, *Bachman* and *Wilson* (1940) obtained a potent substance in a highly purified form from the urine of pregnant women by adsorption on finely divided benzoic acid, further fractionating with ethanol, and by shaking the water-soluble precipitate with chloroform. This procedure does not injure the hormone to an appreciable extent, but removes protein impurities which are readily denaturated by this treatment. Using this procedure *Gurin* and his coworkers (*Gurin et al.* 1940 a) obtained fractions with a maximal biological activity of 6—8,000 I.U. per mg. Electrophoretic studies on these fractions showed that the hormone is very nearly homogeneous (*Gurin et al.* 1940 a and b).

Katzman, *Godfrid*, *Cain* and *Doisy* (1943) obtained fractions with the same biological potency by chromatographic adsorption on permutit followed by elution with 10 per cent ammonium acetate in aqueous ethanol. These highly purified fractions probably contain the hormone in a pure form which, however, could not be obtained in a crystalline form.

To isolate the chorionic gonadotrophic hormone from the urine of pregnant women, in our experiments the hormone was adsorbed on benzoic acid and the precipitate obtained fractionated with ethanol. During the course of the further purification we have employed *Kossels* discovery (1894) that under special conditions proteins may be precipitated by prota-

mine. It was found namely that the active hormone is precipitated from urine by protamine, treated directly. On the contrary by previous adsorption on benzoic acid and fractionating with ethanol only closely connected protein impurities are precipitated with protamine. This latter treatment made it possible to eliminate the greatest amount of the still remaining impurities and then by the separation of the excess of protamine and the last traces of impurities with Reinecke salt, the hormone was obtained in a crystalline form, assaying 6—8.000 I.U. per mg.

Our procedure is briefly as follows: The urine of pregnant women (1—3 month of pregnancy) adjusted to pH 3.5 with glacial acetic acid is filtered. After filtration the pH is increased to pH 5.0 with sodium hydroxide. A saturated benzoic acid solution in acetone is then added and the mixture vigorously stirred. By this procedure the hormone is adsorbed on the finely divided benzoic acid. The same good results may be obtained by replacing the benzoic acid with stearic or palmitic acid. Advantage can be taken of the fact that on stearic or palmitic acid a considerably smaller amount of the coloured urinary impurities is adsorbed than on benzoic acid. The mixture is allowed to stand for 24 hours, then filtered and the precipitate extracted with cold acetone. It must be noted that this and all the following steps are carried out at a temperature of about $+4^{\circ}$ C. in the refrigerator room. After extraction with acetone the residue is taken up in a M/15 acetate buffer of pH 4.8. To this extract ethanol is then added up to a concentration of 85 per cent. The mixture is allowed to stand for 24 hours, then centrifuged, reextracted with buffer and precipitated again with 85 per cent ethanol. After repeated centrifugation the precipitate is washed with absolute ethanol and dried in vacuo. This extraction procedure yields amounts of 25—30 mg. per litre of urine. (Potency: 4—6.000 I.U. per mg.)

In the next step 0,5 g. of this preparation is dissolved in 50 ml. M/75 phosphate buffer pH 7.4. If any residue remains it is centrifuged off and washed in a few ml. of phosphate buffer. After repeated centrifugation the residue is discarded, the two liquids are combined and 10 per cent aqueous protamine

sulfate solution is added drop by drop. In order to avoid the use of protamine in great excess it is useful to determine by nephelometric titration on a small sample the amount of protamine necessary for the precipitation.

The mixture is allowed to stand for 24 hours in the refrigerator room and then centrifuged, and washed with a few ml. of phosphate buffer. The undissolved residue does not possess any biological activity. Upon the addition of a saturated aqueous Reinecke salt solution in excess to the clear centrifugate the excess protamine and the last traces of impurities are removed. These are centrifuged off. The clear supernatant is now adjusted to pH 3.5 by the addition of dilute sulfuric acid. Accompanied by vigorous shaking ethanol containing 5 per cent m-cresol is added to the solution drop by drop up to a final ethanol concentration of 50 per cent. After two hours' standing this is increased to 60 per cent. Upon the addition of the ethanol the hormone is separating and after 24 hours the formerly amorphous substance begins to crystallise in fine thin colourless needles. After a further 24 hours the crystals are centrifuged off and dissolved in 20 ml. of distilled water. The solution is dialysed over a period of 24 hours in running

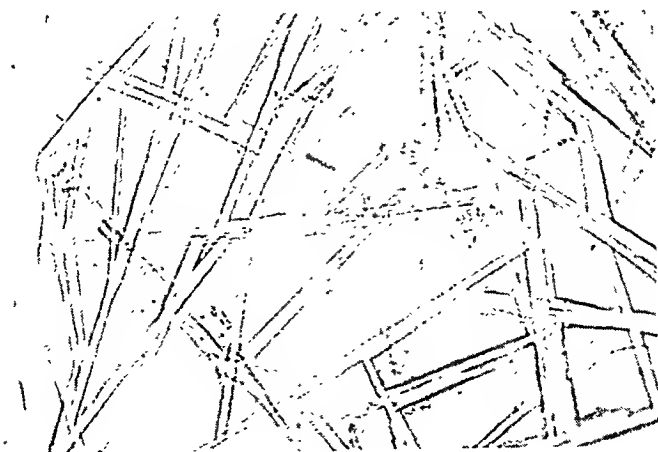


Fig. 1.

Crystals of human chorionic gonadotrophin.
250 x.

distilled water and then ethanol containing 5 per cent m-cresol is added in the described way drop by drop up to a final concentration of 50 per cent. After an interval of two hours the ethanol concentration is increased up to 60 per cent. The solution is allowed to stand for 24 hours and then the crystalline substance is centrifuged, carefully washed with ethanol and moist ether, and dried in vacuo. By microscopical examination the crystals may be recognized as long thin rods or needles. (See figs. 1 and 2.) By combustion an ash-content of 0.3 per cent was found.

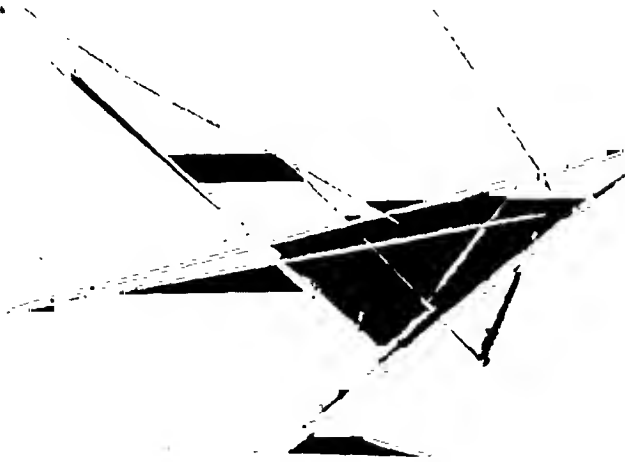


Fig. 2.
Crystals of human chorionic gonadotrophin. Crossed Nicols.
70 x.

By the aid of the procedure described above for the isolation of chorionic gonadotrophin from the urine of pregnant women, crystalline fractions of the hormone may be obtained. By means of electrophoretic studies, using phosphate buffer of pH 7.0 and ionic strength of 0.1, in contrast to the several different fractions previously obtained only one component could be demonstrated, even after 2 hours.

Animal Experiments.

During the course of the isolation of the crystalline and electrophoretically homogeneous chorionic gonadotrophin every single step was controlled in animal tests for biological activity. Three weeks old infantile female white mice, weighing about 6—8 g. were used as test animals. The substance to be tested was dissolved in saline or distilled water and the solution divided into 4 equal doses, which were administered to the animals over three days. On the fifth day after the first injection the animals were sacrificed and the ovaries histologically examined. Positive result was indicated by the presence of corpora lutea. By the assay of the biological activity of the crystalline hormone — using groups of 24 animals for every dose — it has been established that the hormone possesses a fixed potency of 6—8,000 I.U. per mg.

In cases of negative tests the ovaries were always characterized by a typical microscopical appearance, described previously by numerous authors, viz. only one, or a very few growing follicles of extremely great size. This fact seems to confirm the assumption that the growth of follicles observed is referred to a synergism between the chorionic gonadotrophin administered and the intact animals' own gonadotrophic hormones, or it is due to an induced hyperproduction of a follicle stimulator by the anterior pituitary of the test animals (*Levin 1944*). The fundamental bases of this hypothesis have been thoroughly studied by *Collip et al (1933)* and *Leonard and Smith (1934)*. Hence experiments based on this theory were carried out by us on hypophysectomized rats, using the crystalline hormone preparation. In the first series a single dose of 600 I.U. or 1500 I.U. was injected subcutaneously and at the same time one of the ovaries was removed. After 48 hours the animals were sacrificed and both ovaries of every test animal comparatively examined. By the histological examination of these ovaries no growing follicles were to be found. In the next series doses of 600 I.U. or 1500 I.U. were administered daily over a period of 6, 8, 10, 12 or 16 days; the first injection was made 2—3 weeks after the hypophysectomy. In spite of administering chorionic gonadotrophin in high

doses from 3600 I.U. up to 24,000 I.U. there was a complete lack of growing follicles and all the small follicles showed signs of atretia. The actually present corpora lutea showed signs of degenerative processes. On the other hand the histological appearance of these ovaries was dominated by a highly developed interstitial gland.

Our experiments upon the action of the crystalline chorionic gonadotrophin on infantile female mice and hypophysectomized rats reported briefly above seem to support the conception advanced formerly in the literature that the principal biological effect of the chorionic gonadotrophin is due to a synergism between this hormone and a follicle stimulator. In order to obtain further evidence to support this assumption, experiments were also carried out on juvenile female rabbits with the crystalline hormone. White angora rabbits, aged 3 months, weighing 1500—1800 g., were treated in groups of 6 animals with a single intravenous dose. The doses were so chosen that every group received four times higher dose than the preceding one. In this way the lowest dose was 0.09 γ and the highest 6000 γ . After 48 hours the animals were sacrificed, both ovaries removed, dissected free from connective tissue and fat, dried between filter papers and weighed. The results are illustrated by the following dose-response curve, (Fig. 3) representing the correlation between the mean weight of ovaries (mean of 12 ovaries) and the amount of hormone administered (expressed in γ). All ovaries of the animals treated with the three lowest doses were unchanged and had nearly identical weights. On the other hand the ovaries of animals treated with higher doses showed a gradually increased weight corresponding to the dose administered. This increase in weight was mainly due to a general intensive growth of follicles. In addition to this several haemorrhagic follicles were to be observed in all ovaries. Both the ovaries and uteri were highly hyperaemized as a consequence of oestrogenic stimulation. As chorionic gonadotrophin, even in very high doses, failed to induce growth or maturation of the follicles in hypophysectomized animals, our results indicate that chorionic gonadotrophin exerts its highest biological activity in cooperation with a follicular stimulator.

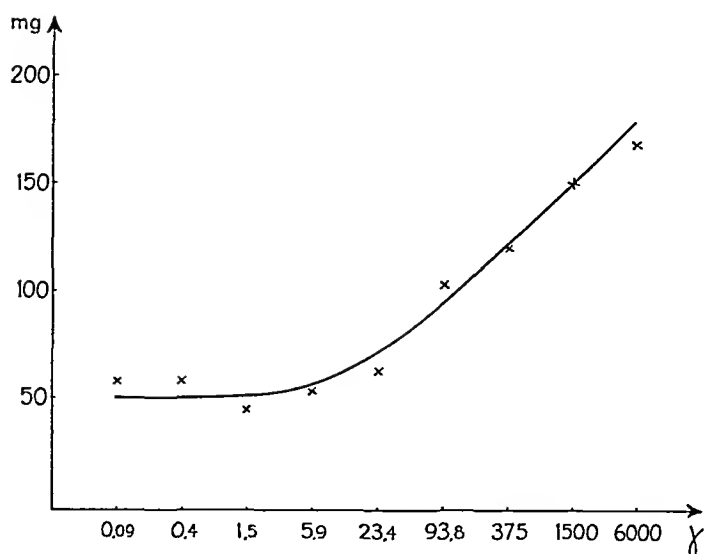


Fig. 3.

Effect of crystalline chorionic gonadotrophin on the ovarian weight of juvenile rabbits. (Doses in γ , mean weight of 12 ovaries.)

CLINICAL OBSERVATIONS

The earliest investigations on the action of chorionic gonadotrophin showed that this substance has a capacity of producing follicular maturation and corpus luteum formation in common laboratory animals, as mice, rats, guinea pigs and rabbits. Thus it seemed probable that the active substance produced by the placenta should be identical with the gonadotrophic hormone of the pituitary. However, in a short time it became clear that this assumption could not be supported by experimental evidence; for in contrast to the hormones of the anterior pituitary and of pregnant mare serum, chorionic gonadotrophin exerted no observable action on the follicular apparatus of hypophysectomized animals. According to these experimental facts, there is a striking difference in the action of these hormones. It was quite a surprising observation that chorionic gonadotrophin had no stimulatory action upon the follicular apparatus of primates. *Engle* (1939) reports that treatment with human chorionic gonadotrophin results only in atretia of

the follicles, while *Hamblen* in his monography (1945) emphasizes that "... chorionic gonadotrophin possesses no important stimulating action upon the ovaries of the higher primate. Its apparency prolongs and enhances the activity of the performed corpus luteum. Its most marked pharmacological action in the female is the depletion of oestrogen level". The stimulatory effect of chorionic gonadotrophin on the corpus luteum was thoroughly studied by *Westman* as early as 1938 and recently by *Braun* and *Bradbury* (1947).

It was rather difficult to understand the great difference in the mode of action of chorionic gonadotrophin on lower laboratory animals and on primates. A presumable explanation might be the difficulty of finding an adequate dosage; it has repeatedly been shown that most of the incomprehensible observations are closely related to difficulties in the dosage. Especially in cases of treatment with chorionic gonadotrophin were these complications dominating; the preparations previously available for intramuscular injections contained impurities and exerted a relatively slight effect. On account of these difficulties, only relatively small doses could be administered and the clinician was often forced to interrupt the treatment because of a high temperature and local reactions of various degrees. It seemed therefore absolutely necessary to have a pure preparation without undesirable side-effects. This long-standing need has now been met by our electrophoretically homogeneous, crystalline chorionic gonadotrophin.

Our clinical studies carried out with the new preparation have been directed

1. to clarify its clinical applicability with special regard to possible toxic side-effects,
 2. to study its action on the structure of human ovaries,
 3. to examine its influence on the excretion of oestrogenic substances, and
 4. to observe its therapeutic effects on patients suffering from amenorrhoea due to pituitary hypofunction.
1. The crystalline hormone was administered to patients first in increasing doses both subcutaneously and intramuscularly, without any observable side-effect. This was followed by intravenous injections in increasing doses up to 12000

I.U. pro die. This dose was repeated daily on three subsequent days, administering in this way a total dose of 36000 I.U. About 30 patients were treated. No side-effects could be observed except in the case of one patient, getting slight shivering and temporarily raised temperature. Considering the mere fact that the hormone is presented in crystalline form, it is likely that possible impurities possessing pyrogen effects may occur only in traces; it must be assumed therefore that the raised temperature was due to an allergic reaction. We have to bear in mind, however, that in rare cases there will always be certain possibilities of such exceptional allergic responses after the parenteral administration of proteins even if highly purified and body-homologous.

Thus it is obvious that this hormone preparation may be used without any inconvenience intravenously as well as intramuscularly or subcutaneously. In this connection it must be emphasized that the highest doses reported above are by no mean the maximal tolerated doses; they may be exceeded without risk, if necessary, but in our material the doses described always elicited the desired effect, making unnecessary the administration of higher doses.

2. The action of the crystalline hormone on the structure of the human ovary was investigated in the following way: A total dose of 36000 I.U. was injected on three subsequent days into ten patients. On operation, which followed 5—7 days after the first injection, 2—7 greatly developed Graafian follicles could be observed in nearly all the ovaries as a consequence of the stimulatory hormone therapy (Figs. 4 and 5). Several of these follicles showed a moderate cystic dilatation, with signs of beginning atretia. (Fig. 6) Thus our investigations provide direct evidence of the fact that even the follicular system of the human ovary can be stimulated by intravenous injections of crystalline chorionic gonadotrophin, when sufficiently high doses are administered. On the other hand no signs of luteinisation could be seen in the growing follicles, and there was a complete lack of any signs of previously ruptured follicles.

3. Having demonstrated the structural changes in the human ovary due to chorionic gonadotrophin, it seemed to be of great

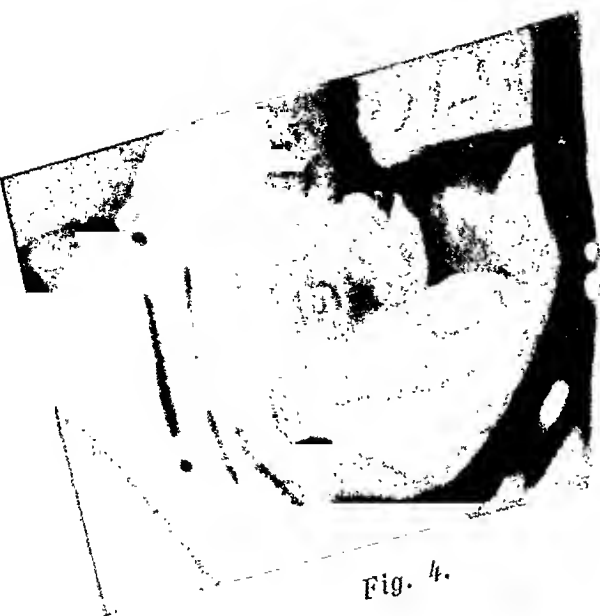


Fig. 4.



Fig. 5.

Ovaries from cases treated with 36000 I.U. crystalline chorionic gonadotropin. Note the intensely growing follicles.

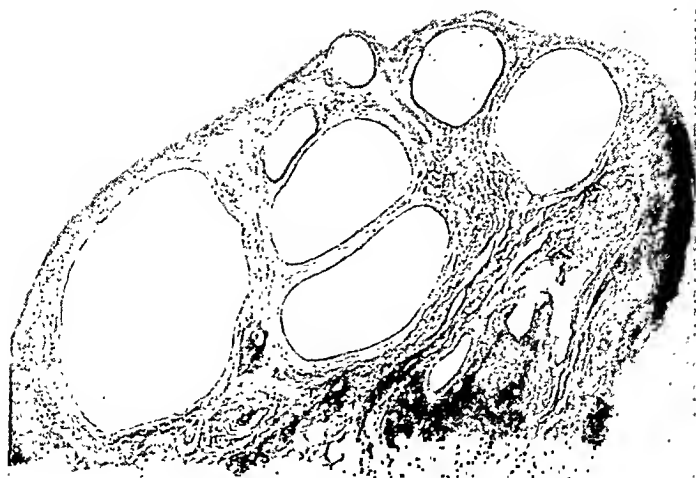


Fig. 6.

Section of ovary from a case treated with 36000 I.U. crystalline-chorionic gonadotrophin. Note marked follicular growth. Haematoxylin-eosin. 4 x.

interest to investigate whether or not there are any correlated changes in the oestrogenic hormone production of the ovaries. The urine of patients treated with crystalline chorionic gonadotrophin was collected over a period of 8 days and assayed for oestrogenic substances. The collection of the total amount of excreted urine started 3 days before treatment and finished 2 days after the administration of the last dose. The results of these assays are mainly uniform; the excretion of the oestrogenic substances increased during the first 24 hours following the first injection, showing afterwards a relatively rapid decrease towards the initial values, which were generally reached on the day of the third injection. The amount of the excreted oestrogens was increased two to ten times as compared to the initial values. In agreement with these results, on operation 4—5 days after the first injection, a marked influence of the oestrogens upon the uterine musculature — manifested in an expressed hyperæmia — could be observed. Thus evidence has been presented that crystalline chorionic gonadotrophin possesses a capacity to stimulate the ovaries to forced production of oestrogenic substances. This forced production, however, has a limited duration, on account of the above-mentioned tendency of the follicles toward atretia.

4. The results of the investigations reported above — highly increased growth of follicles and forced oestrogenic hormone production induced by the crystalline chorionic gonadotrophin — gave a well founded reason for the treatment of patients with amenorrhoea probably due to pituitary hypofunction. The action of the reduced amount of gonadotrophic hormone produced by the pituitary in these patients, was strengthened by the intravenous administration of high doses of crystalline chorionic gonadotrophin. The synergism between the injected chorionic gonadotrophin and the patients' own pituitary hormones resulted in an evident stimulation of the ovaries, which was indicated by induced bleeding from the active endometrium.

The positive results of this treatment in amenorrhoeic conditions support the assumption that the action of the injected

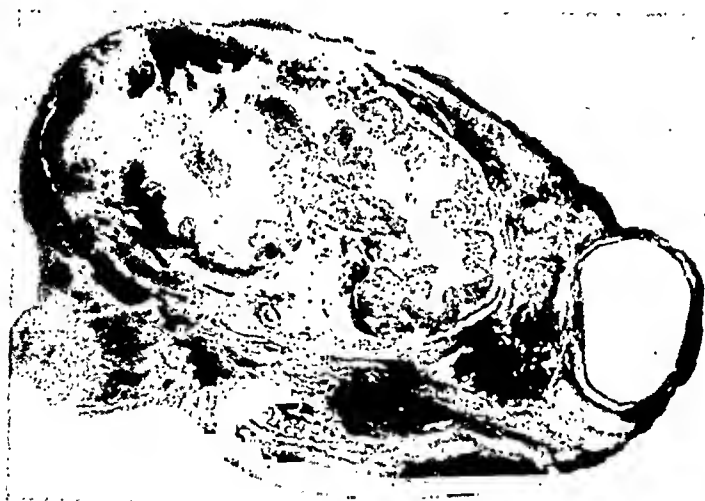


Fig. 7.

Section of ovary from a case treated with 36000 I.U. crystalline chorionic gonadotrophin combined with 3000 I.U. pregnant mare serum gonadotrophin (Antex Leo).
Newly formed corpus luteum as response to the combined treatment. Haematoxylin-eosin. 3 x.

chorionic gonadotrophin combined with that of the insufficiently functioning pituitary may give rise to a gonadotrophic effect of complete value.

Hence when chorionic gonadotrophin is combined with relatively small amounts of pregnant mare serum gonadotrophin

(follicle stimulating hormone) it may be expected that the cooperating hormones will induce ovarian changes analogous to those produced by a normal pituitary under physiological conditions, i.e. rupture of the follicle and corpus luteum formation. On the basis of this assumption injections of crystalline chorionic gonadotrophin were administered in a total dose of 36000 I.U. in combination with 3000 I.U. pregnant mare serum gonadotrophin (Antex Leo). On operation 6—7 days after the



Fig. 8.

Section of ovary from a case treated with 36000 I.U. crystalline chorionic gonadotrophin combined with 3000 I.U. pregnant mare serum gonadotrophin (Antex Leo). Note expressed growth of follicles. (Compare with Fig. 6.) Haematoxylin-eosin. 2 x.

first injection of chorionic gonadotrophin, all the changes expected have been verified (Fig. 7). The growth of the Graafian follicles was highly expressed (Fig. 8). It may be of special interest that in these cases the follicles were surrounded by normal growing granulosa cells and theca interna. (Fig. 9.) No signs of atretia were present. On account of these findings it must be stated that there is a significant difference between these effects and those due to chorionic gonadotrophin by itself, as in the latter case the stimulated follicles in a short time undergo atretia.

Moreover the presence of newly formed intensively vascularized corpora lutea indicates the success of this therapy, provoking rupture of follicles. The results obtained by treat-



Fig. 9.

Normally growing granulosa cells and theca interna from the same ovary as in Fig. 8. Haematoxylin-eosin. 128 x.

ment with massive doses of chorionic gonadotrophin combined with small doses of pregnant mare serum gonadotrophin seem strongly to indicate that chorionic gonadotrophin exerts its maximal effect in synergism with a follicle stimulating factor, and that the latter can be successfully administered in the form of pregnant mare serum gonadotrophin.

DISCUSSION

The results of the electrophoretic investigations on the crystalline chorionic gonadotrophin, reported in this paper, indicate the high degree of purity of the preparation. Detailed analytical results and some more physicochemical properties of the hormone will shortly be published in a following paper. It must here be pointed out that in the case of non crystalline and electrophoretically unhomogeneous preparations (ex. Gurin et al. 1940 a and b; Katzman, Godfrid, Cain and Doisy 1943) the biological activity should by no means be considered as a degree of purity, as the possibility of an eventual synergism between the hormone and the impurities present could not with certainty be excluded.

On the other hand the investigations reported above present sufficient evidence from chemical point of view that only traces of impurities may be present in the crystalline hormone. This assumption is further supported by the fact that the crystalline hormone invariably showed a fixed activity assayed on mice. The isolation of chorionic gonadotrophin in electrophoretically homogeneous, crystalline form has thus led to a preparation possessing constant and well defined biological activity per mg.

Concerning the mode of action of the crystalline chorionic gonadotrophin, our animal experiments confirm the previous results (*Collip et al.* 1933; *Leonard and Smith* 1934). The experiments carried out on hypophysectomized rats, reported in this paper, show that the daily administration of even an amount of 1500 I.U. crystalline chorionic gonadotrophin over a period of 16 days fails to induce any growth or maturation of the follicles. On the other hand a forced follicular growth, followed by corpus luteum formation, always took place when the crystalline preparation was injected to intact animals. Thus the results obtained by us indicate a possibility of investigating — under better defined conditions than previously — the mode of action of chorionic gonadotrophin with special regard to the formation mechanism of oestrogens and to the synergistic action of the anterior pituitary on the follicular growth. Some investigations dealing with these questions are in progress in our laboratories.

The clinical results presented by us clearly show that intravenously injected crystalline chorionic gonadotrophin is well tolerated, without any side-effects, even in daily doses of 12000 I.U. administered on three subsequent days.

In all the cases intravenously treated with crystalline chorionic gonadotrophin marked stimulation of the ovaries was noticed. This stimulatory effect manifested itself in a forced production of oestrogenic hormones coinciding with a highly increased follicular growth. The increased production of oestrogens seems to influence the pituitary, acutely depressing the production of its follicular stimulating hormone. As a consequence the follicles cease developing and the great Graafian follicles then show signs of atretia. However, the increased

production of oestrogenic substances due to the action of the chorionic gonadotrophin seems after the first acute depression to induce a clear stimulation of the pituitary body, as by the administration of high doses of crystalline chorionic gonadotrophin to amenorrhœic patients, we succeeded in provoking rythmical bleedings from a progestional endometrium in the secretory phase. This phenomenon signifies that a spontaneous cycle was induced. If these preliminary results could be verified on a greater material, it would mean a new pathway to the effective treatment of amenorrhœa related to pituitary hypofunction. Furthermore it seems to be obvious from the investigations described above that combined treatment with pregnant mare serum gonadotrophin specially increases the chances of a successful therapy in amenorrhœa. Still more promising results may be expected if pregnant mare serum gonadotrophin will also be available in a pure form. For the isolation of this hormone experiments are going on in our laboratories.

As can be seen from this short discussion, the reported results concerning the clinical application of crystalline chorionic gonadotrophin have clarified in broad outlines the problems connected with the mode of action of this hormone. However, the limited number of cases hitherto observed makes it hardly possible to analyse these problems in detail. Further experiments on the rôle of the crystalline chorionic gonadotrophin in the relationship between gonads and the hypophyseal-diencephalic system and a critical review of the corresponding literature will be published later.

SUMMARY

1. A method is described for the isolation of chorionic gonadotrophin from the urine of pregnant women in crystalline and electrophoretically homogeneous form.
2. This crystalline preparation possesses a constant biological activity of 6—8000 I.U. per mg.
3. The crystalline chorionic gonadotrophin shows a marked stimulatory action on the growth and maturation of the follicles and on the formation of corpus luteum in intact mice, rats and rabbits, but fails to do so in hypophysectomized rats.

In this latter group the crystalline hormone produces only an extensive development of the ovarian interstitial gland.

4. The intravenously administered crystalline chorionic gonadotrophin is well tolerated by patients even in daily doses as high as 12000 I.U. injected on three subsequent days.

5. Intravenously administered crystalline chorionic gonadotrophin gives rise to an increased follicular growth in the human ovary and to a forced production of oestrogenic hormones. The increased follicular growth is soon followed by atretia of the greatest Graafian follicles.

In the treatment of amenorrhœa due to pituitary hypofunction, high doses of intravenously administered crystalline chorionic gonadotrophin may induce bleedings from the progesterional endometrium. This fact underlines the importance of the crystalline preparation as a therapeutic agent in amenorrhœa due to pituitary insufficiency.

7. Crystalline chorionic gonadotrophin combined with small doses of pregnant mare serum gonadotrophin (follicle stimulating hormone) produces an intensive development of the follicles. The granulosa and theca cells do not show any signs of degeneration. Follicular rupture and corpus luteum formation takes place in contrast to the effects induced by the action of crystalline chorionic gonadotrophin administered alone.

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From the Hormone Department
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NORMAL URINARY EXCRETION OF NEUTRAL 17-KETOSTEROIDS WITH SPECIAL REFERENCE TO AGE AND SEX VARIATIONS*)

BY

CHRISTIAN HAMBURGER

The colorimetric determination of 17-ketosteroids in the urine by means of the *Zimmermann reaction* has been employed extensively as a diagnostic adjuvant in clinical medicine. Because of the accuracy and simplicity of this chemical reaction, in most places it has replaced the circumstantial, space-requiring and not very accurate biological test (the capon comb test).

The steadily increasing number of clinical hormonal analyses that have been carried out in the *Hormone Department of the State Serum Institute* made it more and more difficult to perform the capon comb test for the androgen content of the urine in a safe and reliable way even though we had several hundred capons at our disposal. In March 1947, the acquisition of a spectrophotometer made it possible for us to adopt the chemical 17-ketosteroid determination completely or in part.

The different biological effects of the 17-ketosteroids in

*) A preliminary account of the investigations was made the subject of a paper given at the meeting of the Danish Society for Endocrinology held on October 1, 1947.

the urine, however, naturally brings about that the chemically measured values will not necessarily be identical with the values found by the biological method. So, on turning from the capon comb test to the colorimetric technique we were not able off hand to make use of our normal material obtained by capon comb tests (*Hamburger, Halvorsen & Pedersen, 1945*) as a basis for comparison in the clinical hormone analyses. We had to obtain a new control material based on chemical analyses. Indeed, this was required in particular, as the literature so far had brought rather scanty reports on fairly large normal materials with regard to the excretion of 17-ketosteroids.

Barnett, Henly, Morris & Warren (1946) have tabulated several normal materials reported from 1939 to 1946, including their own cases, but the materials presented by the individual laboratories comprised merely between 9 and 24 individuals of either sex. Among the authors not included in this schematic survey, mention is to be made of *Scott & Vermeulen* (1942), *Talbot & Butler* (1942) and *Venning & Kazmin* (1946). In most of these materials the younger and middle-aged individuals were found to show an average 24-hour excretion of approximately 13 mg. for men, and 9 mg. for women, whereas *Luft* (1943) found considerably higher values for both sexes. Recently a large material has been reported by *Forbes, Donaldson, Reifenstein & Albright* (1947), who in 73 young men found the average excretion to be 12.5 mg. per 24 hours, in 65 young women 8.2 mg. As to the limits, lower and upper, for the average normal excretion in the materials mentioned, they may roughly be set at one-half and the double of the average. The largest material of children has been reported by *Nathanson, Towne & Aub* (1941), who found very low values in children under 9 years, then an increase during puberty with somewhat higher values for boys than for girls — findings that have been confirmed by later investigators. As to the excretion of 17-ketosteroids in old people, various reports of a few cases have been published now and then, showing considerably lower values than found for the younger individuals. *Hamblen et al.* (1939), however, found the excre-

tion to be higher for women during and after the climacterium than for younger women. Still, so far no systematic investigation into the variations of the 17-ketosteroid excretion in the various age-classes appears to have been carried through.

The main purpose of the present studies has been to obtain a normal material ranging from childhood to senescence, and sufficiently large for division into age-classes.

MATERIAL AND TECHNIQUE

Urine. — The specimens of urine analyzed here originate exclusively from apparently healthy men and women giving a negative history as to present or recently passed diseases. Thus we have refrained from using any specimens of urine obtained, for instance, through surgical clinics from otherwise healthy persons who had suffered some fracture or other accidental injury, because *Forbes et al.* (1947) were able to demonstrate that any form of traumatic injury may influence the 17-ketosteroid excretion (cf. *Selye's* »general-adaptation-syndrome«). A total 24-hour specimen of urine was obtained from each individual, precise instructions being given as to the collection of the urine. In a few cases the 24-hour output of urine was collected daily for some length of time in order to estimate the individual variations in the 24-hour excretion of 17-ketosteroids.

Extraction and Purification of the Extracts. — Combined acid hydrolysis and benzene extraction was employed. The residue from the benzene extract was dissolved in ether, from which the neutral 17-ketosteroids were separated by shaking with NaHCO_3 , NaOH and water. The ether was evaporated, and the residue was dissolved in absolute ethyl alcohol (in an amount corresponding to one 24-hour output of urine in 40 ml.).

Chemical Reaction. — We have employed the *Callow* modification of the *Zimmermann* reaction, following strictly the directions given by *Callow, Callow & Emmens* (1938).

Colorimetric Assay. — This is made by means of a *Coleman*

spectrophotometer (Junior model A) with two six-volt accumulators for source of current. The original round 19-mm. cuvettes (No. 6—302) were used. In the normal material which will be presented here the analyses were made in triplicate, and the absorption was measured at 10 different wave-lengths, distributed evenly from 400 to 700 m μ . (This was done with a view to investigations of the validity of the application of color correction equations. Routine assays were performed in duplicate, and the absorption was measured only at 530 and 470 m μ). For the sake of control, a test with 0.10 mg. androsterone was made daily.

Calibration Curve for Androsterone. — In tests with doses of up to 0.15 mg. androsterone the calibration curve is found to form a straight line. Among 223 tests with 0.10 mg. androsterone only four measurements fell outside the range of D 0.59—0.70 and the average D value (= »optical density«) was 0.65.

CORRECTION FOR UNSPECIFIC CHROMOGENIC COMPOUNDS

The absorption curves throughout the visible spectrum give a good expression for the more or less pronounced deviation of the urine extracts from the pure 17-ketosteroids with reference to the quality of the reaction color. The tendency of the urine extracts to give color tones with a brownish tint is largely due to unknown chromogenic urinary elements, although also 3-ketosteroids and 20-ketosteroids may contribute to the unspecific color reaction (*McCullagh, Schneider & Emery, 1940*).

Fig. 1 shows three examples of absorption curves from our normal material, namely: the purest urine extract (K 5 a), a fairly pure one (K 115), and a very unspecific one (K 122), together with the curve for the non-ketonic fraction from the *Girard* process. Besides, in this graph three average curves have been drawn for different androsterone concentrations (0.75, 0.50 and 0.25 mg./ml.). It will be noticed that the ab-

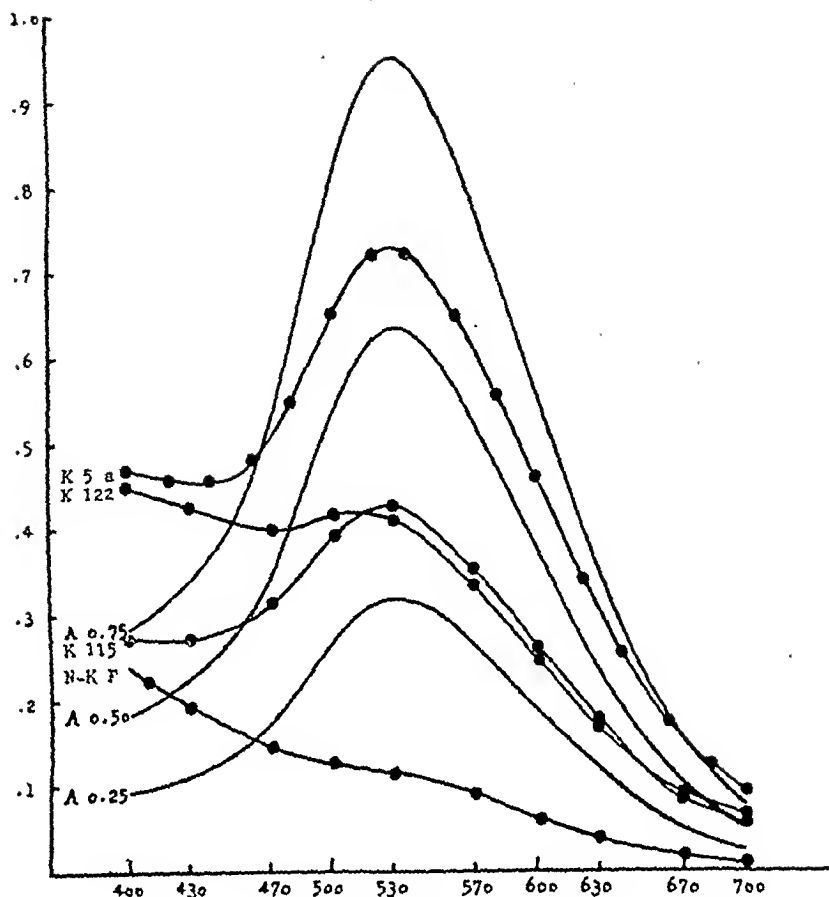


Fig. 1.

Absorption curves for androsterone (0.75, 0.50 and 0.25 mg./ml.) three different urine extracts (K 5 a, K 122 and K 115), and the non-ketonic fraction from the Girard process (N-K F).

. Ordinate: optical density. Abscissa: wave length in mμ.

sorption maximum for pure 17-ketosteroids is found at 530 mμ, whereas the non-ketonic fraction has its maximum in the extreme violet field, and — with regard to the form of their curves — the urine extracts fall between these two extremes.

While the form of the absorption curves for the urine extracts is subject to wide variation from one individual to another, the curves remain strikingly constant for one and the same person if the urine is examined at intervals of weeks or even several months. So, in the individual person, the pro-

portion between the amounts of 17-ketosteroids and unspecific chromogenic urinary elements must be fairly constant.

A numerical expression for the deviation of the urine extracts from the values for pure 17-ketosteroid solutions can be obtained by calculating the proportion between the absorption in the blue (or violet) field and in the green zone. For 17-ketosteroids we have found $D\ 470/D\ 530 = 0.60$, for the non-ketonic fraction $= 1.27$ while the quotient for most of the urine extracts falls between 0.70 and 0.90, on an average higher in extracts with a low 17-ketosteroid content. (The three urine extracts in Fig. 1 showed a quotient of 0.66, 0.75 and 0.98, respectively).

As an attempt to avoid the overestimation of the 17-ketosteroid content of the urine extracts resulting from the presence of unspecific compounds, we have treated a number of urine extracts, differing greatly in their 17-ketosteroid content after the *micro-Girard process* given by *Pincus & Pearlman* (1941), or by absorption to MgO (*Bowman*, 1945). We found the percental yield to vary with the amount of 17-ketosteroids in the extract, and the separation of the ketonic and the non-ketonic fractions was incomplete and inconstant, so that these methods had to be looked upon as rather unsuitable, besides being all too circumstantial for clinical routine analyses.

So, for elimination of the influence of the unspecific chromogenic substances upon the measurements of the reaction, to us it seemed preferable to use a color correction equation. Various methods of calculation have been suggested for this purpose, all being based upon the proportion between the absorption at 520—530 m μ and at lower wave lengths. The accuracy of this correction method may be controlled by adding varying, known, amounts of 17-ketosteroids to urine extracts with a very low 17-ketosteroid content or, preferably, to the non-ketonic fraction and then submit the measurements obtained to the corrective calculation. The method which in our hands has given the best results is the formula worked out by *Gibson & Evelyn* (1938) —and, as far as that goes, this

formula also appears to be the one employed most frequently. Thus, the serviceability of this formula has been tried out thoroughly and described by *Fraser et al.* (1941) and *Talbot, Berman & MacLachlan* (1942). Adapted for the 17-ketosteroid determinations the formula of *Gibson & Evelyn* is:

$$g_k = \frac{(K_i \times g_e) \div b_e}{K_i \div K_s},$$

in which g_k = the corrected absorption measurement in green light, and $K_i = \frac{b_i}{g_i}$ = the proportion between absorption in blue light and in green light for the non-ketonic fraction; $K_s = \frac{b_s}{g_s}$ = the same proportion for pure 17-ketosteroids; g_e = absorption of urine extract in green light, and b_e = absorption of urine extract in blue light.

As mentioned above, we have found $K_s = 0.60$, and on the basis of measurements on the non-ketonic fraction (*Girard*) from 15 urine extracts from 10 men and women in different age-classes we found $K_i \approx 1.27$. In order to avoid all calculations we have constructed a *nomogram*, presupposing the above mentioned values for K_s and K_i , and further assuming that 0.10 mg. 17-ketosteroids gives a D value of 0.65 at 530 $m\mu$, and that the amount of urine extract employed for the reaction corresponds to 1/200 of the 24-hour urine. As the *principle* of this nomogram may reasonably be looked upon as serviceable to other laboratories too, it is given here (Fig. 2). The corrected 17-ketosteroid value (recorded in mg./24-hours) is found as the point where a straight line through the values obtained for D 530 and D 470 intersects the third line.

It is to be emphasized that all color corrections give merely approximately correct values, as they presuppose the unspecific chromogenic compounds of different urine extracts to have the same absorptive aspects as the non-ketonic fraction — something we hardly may take for granted. But there can be

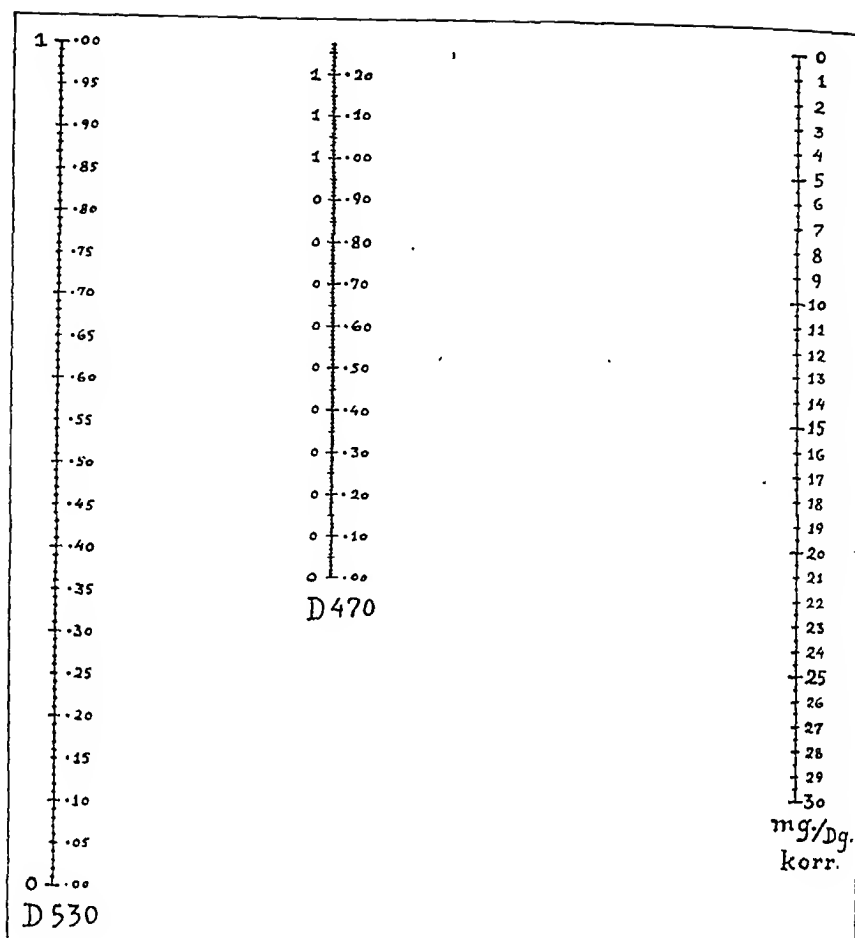


Fig. 2.

Color correction nomogram, based on Gibson & Evelyn's formula. Presuppositions: D 470/D 530 for pure 17-ketosteroid = 0.60, and D 470/D 530 for the non-ketonic fraction = 1.27, besides D 530 for 0.10 mg. 17-ketosteroids = 0.65, and provided that the reaction is performed with an amount of extract corresponding to 1/200 of the 24-hour urine.

no doubt that the corrected values thus obtained will come nearer the true value than the uncorrected, and in practice this form of correction has proved highly serviceable.

VARIATIONS IN THE 17-KETOSTEROID OUTPUT FROM DAY TO DAY.

When we are to estimate the analytical findings in the urine, the question suggests itself at once as to how much the output may vary from day to day in the same person. *Werner* (1943) investigated the 24-hour output of 17-ketosteroids in 5 young men for some months and found deviations from the mean as high as up to 50 per cent. *Venning & Kazmin* (1946) examined the urine of 3 men and 1 woman through 2 weeks and found the limits for the 17-ketosteroid excretion in these subjects to be respectively 16—29, 10—18, 12—19 and 14—27 mg./24-hours (calculated on the basis of 48-hour specimens).

We have tried to elucidate this question through examination of two normal men and two normal women, the analyses being performed partly on single 24-hour specimens at intervals of months, partly by continued daily assays through 1 or 2 weeks. The results are recorded in Table 1, from which

it is evident that the dispersion ($S = \sqrt{\frac{\sum d^2}{n \div 1}}$) for the daily variations is respectively: 2.1, 1.4, 1.4 and 1.5. As there is no statistically significant difference between these four dispersions, the total dispersion has been calculated and found to be 1.76 [f (= degrees of freedom) = 45] with an average 17-ketosteroid content of 12.0 mg./24-hours.

In contrast to most other investigators, *Hollander, Kriss, Klempner & Frank* (1943) found the daily variations (for the androgen content of the urine, estimated biologically) to be smaller when the content was calculated per liter than per 24-hours. No decisive answer to this question is afforded by the present material, as the two men showed no relation between the 17-ketosteroid output and the volume of the 24-hour urine (see Table 1) whereas in the 2 young women there appears to be some relation between the two values. Indeed, there will be no particular reason for calculating the output per liter unless the 17-ketosteroid excretion is proportional to the 24-hour output.

Table 4.

Variations in the 24-hour Output of 17-ketosteroids in 2 Normal Men and 2 Normal Women.

Date	24-hour urine	17-keto-steroids mg/24 hrs.	Deviation from the mean value	Date	24-hour urine	17-keto-steroids mg/24 hrs.	Deviation from the mean value
<i>Man, aged 43 years</i>				<i>Woman, aged 23 years</i>			
12/3-47	850	15.0	+ 1.7	23/3-47	1020	10.3	+ 0.5
10/4	1225	14.8	+ 1.5	30/4	740	8.9	- 0.9
11/4	1400	12.0	- 1.3	14/5	670	9.8	0
12/4	980	13.5	+ 0.2	27/8	610	10.2	+ 0.4
13/4	1140	10.5	- 2.8	24/10	590	7.9	- 1.9
14/4	1030	11.0	- 2.3	25/1-48	660	11.0	+ 1.2
15/4	930	11.6	- 1.7	26/1	580	9.0	- 0.8
16/4	900	15.3	+ 2.0	27/1	440	9.2	- 0.6
17/4	1040	11.4	- 1.9	28/1	535	9.5	- 0.3
18/4	950	13.0	- 0.3	29/1	760	12.3	+ 2.5
19/4	1050	15.8	+ 2.5	30/1	500	7.6	- 2.2
20/4	980	9.5	- 3.8	31/1	980	11.4	+ 1.6
21/4	1080	10.4	- 2.9	Mean value 9.8 S = 1.4			
22/4	1060	12.8	- 0.5				
27/1-48	1100	15.9	+ 2.6				
28/1	1170	15.1	+ 1.8				
29/1	1120	12.9	- 0.4				
30/1	1220	16.8	+ 3.5				
31/1	1140	15.1	+ 1.8				
1/2	1180	14.6	+ 1.3				
2/2	1190	11.9	- 1.4				
Mean value		13.3	S = 2.4				
<i>Man, aged 36 years</i>				<i>Woman, aged 18 years</i>			
25/3-47	1370	14.0	- 0.6	17/4-47	880	8.0	- 1.2
23/1-48	1500	15.9	+ 1.3	29/1-48	790	8.3	- 0.9
24/1	1230	17.5	+ 2.9	30/1	700	8.3	- 0.9
25/1	1065	14.0	- 0.6	31/1	650	7.9	- 1.3
26/1	1680	14.6	0	1/2	870	9.7	+ 0.5
27/1	1320	14.2	- 0.4	2/2	900	12.2	+ 3.0
28/1	930	12.9	- 1.7	3/2	820	10.1	+ 0.9
29/1	1740	13.9	- 0.7	4/2	1120	8.9	- 0.3
Mean value		14.6	S = 1.4	Mean value		9.2	S = 1.5

In our material recorded in Table 1, as a matter of fact the total dispersion is but very little greater than the dispersion in the technical variation. So, the possibility cannot be refuted that the daily variations demonstrated here may largely be due to the technical variation.*)

DEPENDENCY OF THE 17-KETOSTEROID OUTPUT ON THE AGE AND SEX OF THE INDIVIDUAL

The main purpose of the present studies has been to ascertain the average 17-ketosteroid output in normal men and women in all age-classes and to establish the upper and lower limits for the normal output, so that this material might be used as the basis for comparison with our 17-ketosteroid assays on urine from patients with various diseases.

The material comprises 137 normal male subjects from 3 to 102 years old and 127 normal females, from 2 to 92 years old. The outcome of this investigation is presented graphically in Fig. 3, which also gives the analytical result from 10 male castrates, who were included in order to obtain information about the part played by the testes in the total 17-ketosteroid output.

From Fig. 3 it will be noticed that, on the whole, the male output keeps at a higher level than the female output, although the values are overlapping rather extensively. From this graph it is further evident that *the output for male castrates is of about the same magnitude as for the women*. Finally, the material indicates that the greatest output takes place at the age 20—30 years.

The average output in male and female subjects is illustrated graphically in Fig. 4. Within each age-class covering 10 years (0—9 years, 10—19 years, and so on) the average age and the average output are plotted in the graph as circles. Then the mean curve is drawn after it has been made sure

*) The statistical calculations are performed in the Statistical Dep. of the State Serum Institute by G. Rasch, Ph. D.

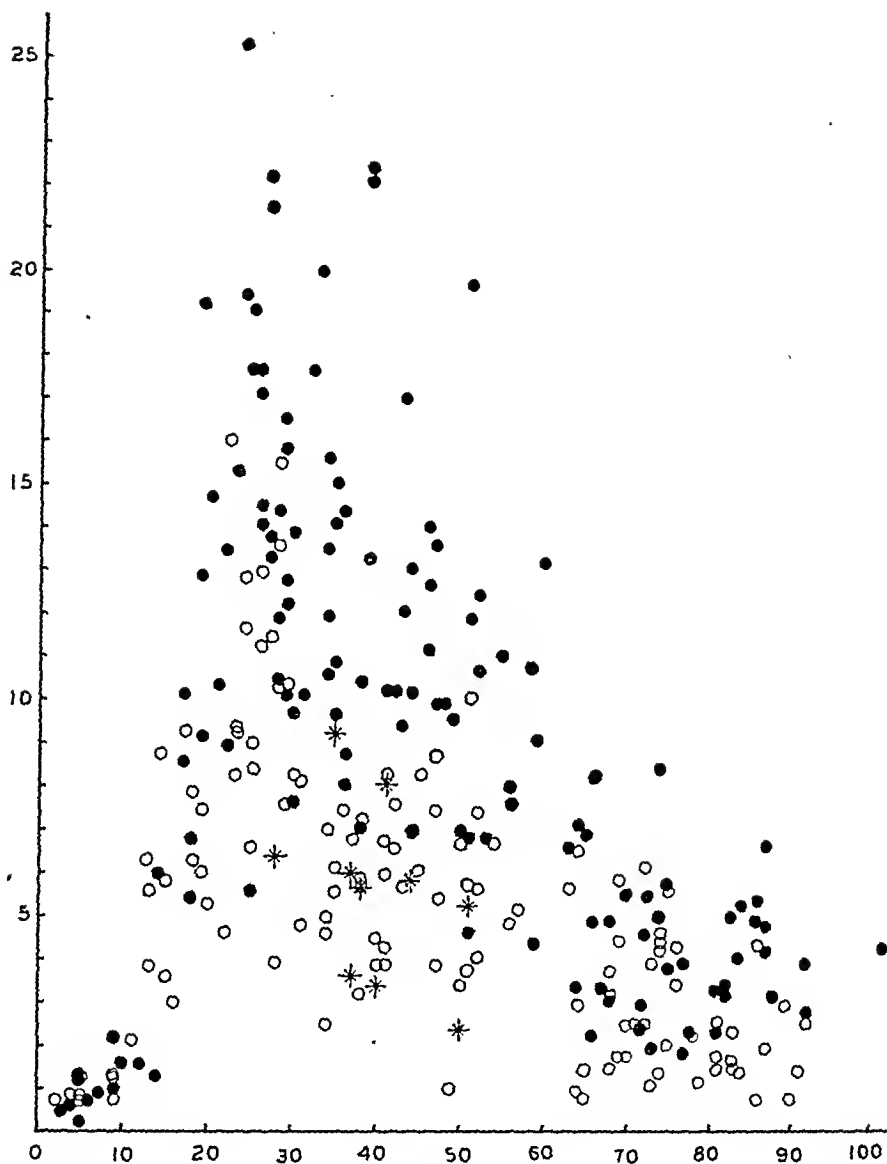


Fig. 3.

24-hour output of 17-ketosteroids in 137 normal male subjects (\bullet), 127 normal female subjects (\circ), and 10 male castrates (\star). Ordinate: mg./24-hours (corr.). Abscissa: age of the subject.

that its form did not change when the average calculation was made on the basis of the age-classes 5—14 years, 15—24 years, and so on. The output of the boys and girls kept parallel until

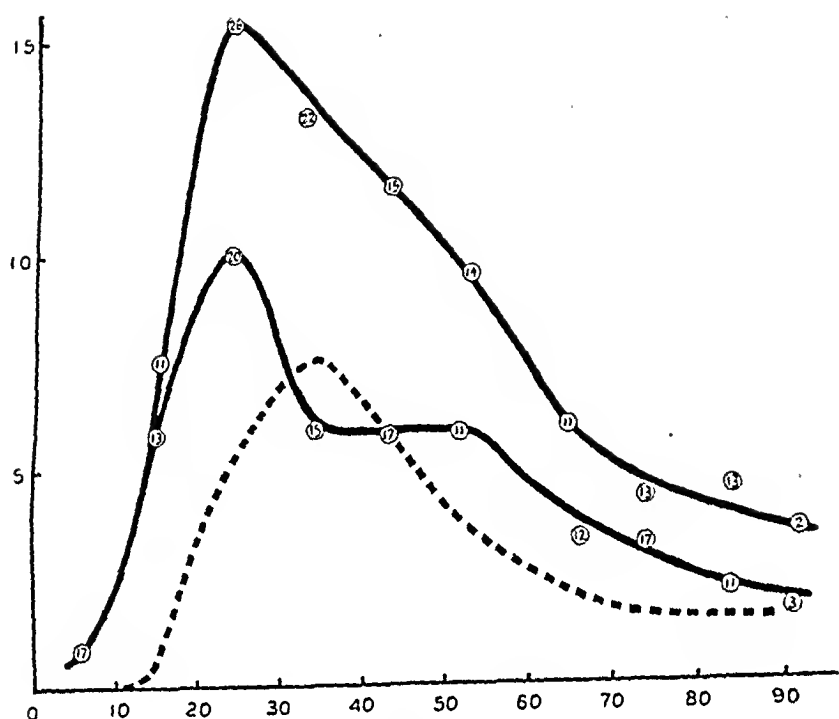


Fig. 4.

Average excretion of 17-ketosteroids per day in normal men (upper full-line curve) and in normal women (lower full-line curve); the figures in the circles give the number of individuals in the age-class concerned. The dotted curve represents the difference between the male curve and the female (= contribution by the testes in the 17-ketosteroid excretion).

Ordinate: mg/24-hours (corr.). Abscissa: age of the subject.

the age of 15 years, whereafter the male curve throughout life kept at a higher level than the female curve. For either sex the maximum falls at the age of about 25, amounting to 15.4 mg./24-hours for the males, and 10.0 mg./24-hours for the females. Through the following decades the men show a gradual fall, while in the women there is a rather steep fall unto the middle thirties, which is followed by a plateau to the age of 50—60, and then by a gradual fall. *Hamblen et al.* (1939) found the 17-ketosteroid secretion to lie at a higher level after the climacterium than before. The authors took this to signify a compensatory hypertrophy of the adrenal

cortex, after the cessation of the hormonal function of the ovaries. A mere glance at Fig. 4 will show that our results positively go against this view.

It is the general consensus of opinions that in men the 17-ketosteroids originate from the testes as well as from the adrenal cortex. Consequently, the difference between the male and female curves will represent the partition of the testes in the 17-ketosteroid output. This assumption is corroborated by the circumstance that we have found the output to be of the same magnitude in women and castrated men. The curve resulting from such a subtraction is shown in Fig. 4 (the dotted curve). It is rather remarkable that this curve takes quite the same form as the average curve previously obtained after the biological method for assay of the normal androgen output in men (*Hamburger, Halvorsen & Pedersen, 1945*); further the increase in the output observed in puberty was seen to continue through adolescence and attain a maximum at the age of 30—40 years. These findings make it even more probable that the *mean curve for the women — at any rate, quantitatively — represents the adrenal cortical excretion of 17-ketosteroids in both sexes*, and that the higher level of the male curve is due to the addition of 17-ketosteroids from the testes. Accordingly, the 17-ketosteroid production from the adrenal cortex would attain its maximum already in the twenties, while the maximal 17-ketosteroid production from the testes, comes about ten years later.

As has been mentioned already, the values obtained with the biological test (capon comb method) and the values obtained through the *Zimmermann* reaction are not necessarily identical. The 17-ketosteroids — among which androsterone, dehydro-*iso*-androsterone and etiocholanolone are most abundant — give the same color development, qualitatively as well as quantitatively in the *Zimmermann* reaction (*Baumann, Metzger & Sprinson, 1942*). We have found that, in biological assays, androsterone is 5 times more active than dehydro-*iso*-androsterone, and etiocholanolone is reported as having no biological effect. Thus, it was not to be expected we might

find any particular agreement between the chemical and biological values obtained unless the mutual quantitative relations between the various 17-ketosteroids were constant. That this is not the case, however, is evident from the studies on the androgen output of the male castrate as determined biologically and the 17-ketosteroid excretion demonstrated chemically. Thus, while normal middle-aged men on an average excrete androgenic substances corresponding to about 50 I. U. of androsterone per day, the male castrates were found to excrete about 10 I. U. per day, making the proportion between the two groups 5:1. In assays by means of the *Zimmermann* reaction we found this proportion to be 2:1. The only reasonable explanation of this difference, must be a considerable difference in the mixture of 17-ketosteroids in the urine. As far as that goes, the slight difference in the 17-ketosteroid output in normal and castrated men has been demonstrated already by *Callow, Callow & Emmens* (1940), but the practical consequences of these observations to the clinical hormonal analyses appear not to have been recognized particularly. For, from these it would be natural to conclude that the biological method, which shows the more pronounced difference between normal and castrate values, will also give a more reliable information about the hormonal function of the testes, and hence it will seem preferable when this information is desired, whereas the chemical method is superior when it comes through hormonal analysis to throw light on the state of the adrenal cortex.

According to some preliminary examinations (*Hamburger*, 1947) the *antimony trichloride reaction* for 17-ketosteroids elaborated by *Pincus* (1943) gives a greater difference between the urines of normal and castrated men than does the *Zimmermann* reaction. So, if these findings be confirmed by a more comprehensive material it may be reasonable to look upon this method as preferable for information concerning the rôle of the testes in the 17-ketosteroid excretion.

In the clinical hormonal analyses, not only the average values for the normal 17-ketosteroid excretion are of interest;

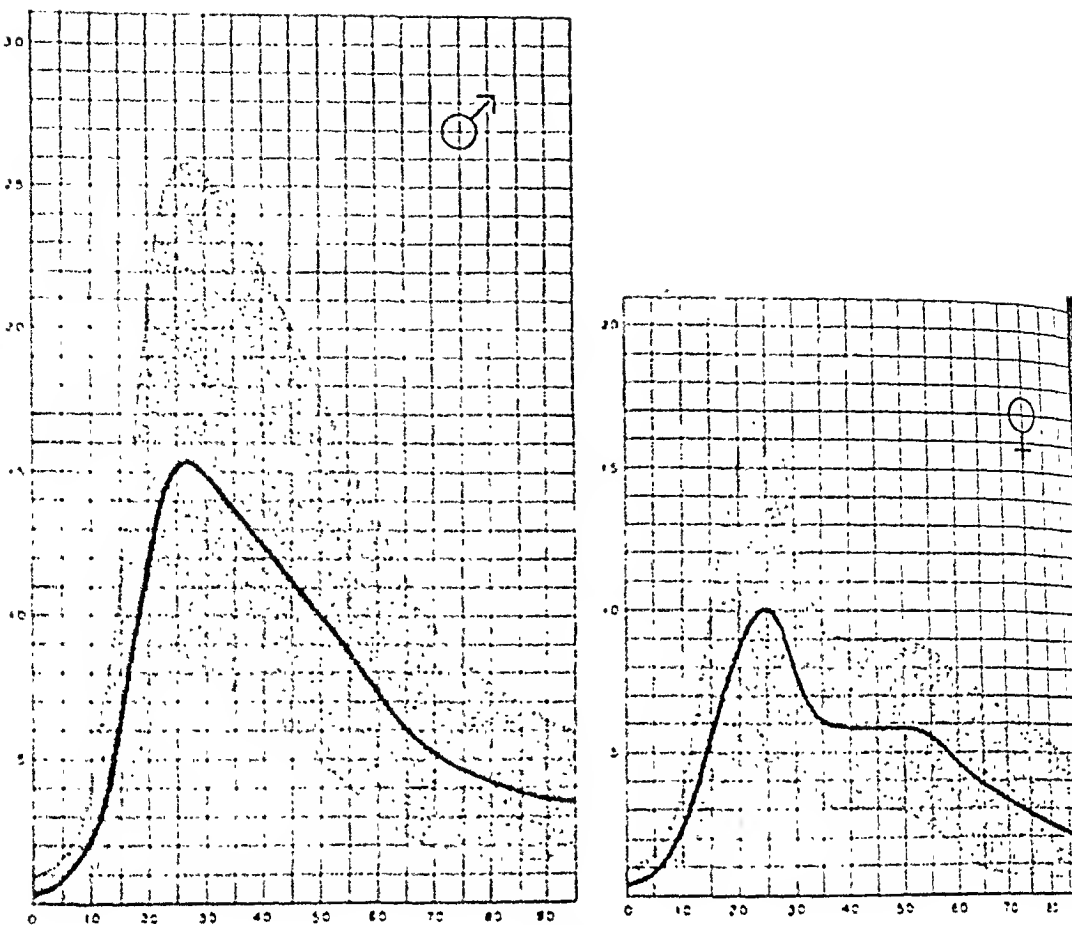


Fig. 5.

Excretion of 17-ketosteroids in normal men and women. The black line shows the average output, and the stippled area gives the zone within which 97-98 per cent of the values in our material have fallen.

Ordinate: mg/24-hours (corr.). Abscissa: age of the subject.

at least the limits, upper and lower, for the output in the different age-classes are equally important. In Fig. 5 the stippled area gives the field within which 97-98 per cent of the values in our normal material have fallen, respectively in male and female subjects.

From this, it is evident that if the upper and lower limits were to be given in numerical values, it would require a rather

extensive tabulation, with the material divided into age-classes of 10 years. This difficulty, for instance, made *Talbot & Butler* (1942) leave out numerical values in the work of the clinical significance of 17-ketosteroid assays, replacing them with symbols for the low, moderate and high excretion as compared to the respective normal values. To us it seemed preferable to reproduce the diagrams from Fig. 5 on our reply-cards and to indicate the value by a pencil-mark. Also when summarizing larger materials concerning the 17-ketosteroid output in pathological conditions, this way of graphical recording of the values obtained has the advantage that it offers a more rapid survey of the findings than does a tabulation.

I wish to give my best thanks to everybody who has helped me in getting the many 24-hour urines; in particular, I am greatly obliged to Miss *Grete Holm* for her valuable technical assistance.

SUMMARY

The *purpose* of these studies has been to obtain a sufficiently large material for elucidation of the *dependency of the 17-ketosteroid output upon the sex and age of the individual* and thus, at the same time to establish a comparative basis for the estimation of the clinical hormonal analyses.

The *material* comprises determinations of the 24-hour output of neutral 17-ketosteroids in 137 normal males, from 3 to 102 years old, and in 127 normal females from 2 to 92 years old; in addition, 10 middle-aged male castrates are included.

Technique: Combined acid hydrolysis and benzene extraction is employed. The 17-ketosteroid content of the urine is assayed by means of the *Callow* modification of the *Zimmermann* reaction. The photometric measurements are made by means of a *Coleman spectrophotometer* (Junior model A). The

values thus obtained are corrected for unspecific chromogenic urinary elements by means of a nomogram, constructed on the basis of *Gibson & Evelyn's* color correction equation.

Results: An account is given of the individual diurnal variations in the 17-ketosteroid output. For both sexes the *average output* was found to rise abruptly during puberty and adolescence, and reaching a maximum at the age of 25 years, amounting to 15.4 mg. per day for the men, 10.0 mg. per day for the women. In the men the output was falling gradually to senescence. In the women there was an abrupt fall in the output from the age of 25 to the middle thirties, followed by a plateau to the age of 50 to 60 years and then a gradual fall. The lower and upper limits for normal output were found in all age-classes to lie respectively at *about* 50 per cent under and *about* 50 per cent over the average output. Comparisons have been made between the normal material thus obtained after the chemical method and the previous normal material determined in this department after the biological method. In addition an account is given of the 17-ketosteroid excretion in male castrates. Finally the theoretical and practical consequences of these comparative studies are discussed.

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OESTROGENIC, ANDROGENIC AND
GONADOTROPHIC SUBSTANCES IN THE URINE
OF NORMAL WOMEN
SEX HORMONE ANALYSES I

BY

K. PEDERSEN-BJERGAARD AND M. TØNNESEN

The following comprises a survey of the excretion of oestrogens, androgens and gonadotrophins in the urine of normal female individuals, children as well as sexually mature women with a normal menstrual cycle, and post-climacterial women.

By normal menstrual cycle we understand a regular cycle of a duration of 25 to 31 days, and a bleeding time varying from 2 to 5 days. The hormone excretion in menstrual disorders will be dealt with in a later publication.

Working independently, *Frank, Frank, Gustavson & Weyerst* and *Loewe* in 1925 published their investigations, made with the aid of the *Allen-Doisy* test, on the presence of oestrogenic substance in blood from women. The following year *Frank & Goldberger* (1926) published a method of preparation for demonstrating oestrogenic substance in the blood. After *Loewe & Lange* (1926) as the first had proved the presence of oestrogenic substances in the urine of normally menstruating women, *Siebke* (1930) and *Frank* (1931) described methods for the constant demonstration of oestrin in the urine of normal subjects. As the content not only of oestrogenic but

also of androgenic substances and gonadotrophin in the blood and urine of normally menstruating women is so small that they can still be determined biologically only after previous extraction or precipitation, thus bringing the particular hormone into a more concentrated solution, these pioneer works, together with *Zondek's* alcohol-precipitating method (1930 II) for demonstrating gonadotrophin in urine, and *Frank, Goldberg & Spielman's* method (1931) for demonstrating this substance in the blood, signified a great advance in clinical hormone analysis, even if the methods in use today are much better than those first described.

TECHNIQUE

1) *Collection of urine.*

In analyses for the three different hormones in the urine of normal, non-pregnant individuals a concentrated extract of the total diurnal output must be prepared, because the hormonal content is so small that a direct injection of the urine will not give a positive result, at any rate not by the biological assay methods available today. What is more, both androgenic and oestrogenic substances in the urine occur to some extent in a coupled, inactive form which must first be split before the full biological expression of the hormone content in the urine is obtainable.

As the results are given in terms of the daily output, which is a more reliable method than recording the output per litre, it is vital that the 24-hour urine should be collected with the greatest care. The collectors were therefore instructed to empty the bladder at the time collection was to begin. The urine was thereafter collected up to and including the last urination exactly 24 hours after the bladder was emptied. The urine was then processed as quickly as possible after collection; in some cases where collection took place in the provinces, a period of up to a week might elapse from the time of collection until the urine could be dealt with. In order to prevent any destructive effect of bacterial infection on the active substances, all flasks of 1000 ml. for urine collection contained an anti-

septic: 5 ml. chloroform + 15 ml. rectified alcohol. Where gonadotrophin and oestrogen analyses were made simultaneously, urine for two full days was collected and, after mixing, divided into two portions and each analysed for its hormone. If simultaneously an analysis was made for androgenic hormone, three days' urine was collected and in the same manner after mixing divided into three equal portions for the determination of each hormone.

2) *Preparation of gonadotrophin extracts.*

The gonadotrophic hormones are precipitated by the method of *Levin & Tyndale* (1936) by adding 20 ml. of a 20 % tannic acid solution per litre urine after adding to the urine 7 ml. acetate acetic acid pH 4.75 per litre. The sediment centrifuged from one day's urine is dissolved in 45 ml. borate buffer pH 9.5 (*Thomsen & Pedersen-Bjergaard* (1936)) after being washed in several portions of absolute alcohol and acetone and dried.

Infantile female rats were used for the tests in the gonadotrophin determinations.

3) *Preparation of androgenic and oestrogenic urine extracts.*

Glimm & Wadehn (1929) were the first to draw attention to the fact that the oestrogenic substances excreted in the urine occur in an ether-soluble and an ether-insoluble form. They converted the ether-insoluble to the ether-soluble form by heating the urine after its reaction had been made acid. They found that the ether-insoluble part represented about 15 % of the physiologically active substance.

Marrian (1930) showed that heating the urine after adding acid brought about an easily demonstrable increase of the oestrogenic properties of the urine. This was subsequently confirmed by *Doisy, Veler & Thayer* (1930) and many others.

After *Adler* (1934) had shown that the androgenic substances in the urine like the oestrogenic substances occur partly in the free form, almost insoluble in water but readily soluble in lipoid solvents, and also that the bound forms can be broken down by heating with the required quantity of a strong acid,

whereupon the liberated androgenic and oestrogenic substances can be extracted with a lipoid solvent, we employed the following procedure for isolating these substances from urine, a procedure which in principle is the same as that described by *Dingemanse, Borchardt & Laqueur* (1937), combining hydrolysis and extraction.

To 24 hours' urine was added 40 ml. concentrated sulphuric acid per 1000 ml. urine; this was followed by extraction by boiling twice with 500 ml. carbon tetrachloride. All carbon tetrachloride was distilled from the extracts under vacuum. The extract thus obtained, containing the androgenic and oestrogenic substances from 24 hours' urine, may now after dissolving in 10 ml. olive oil be titrated biologically for its content of oestrogenic substances on spayed mice and after dissolving in 10 ml. alcohol for its content of androgenic substances on capons.

4) *Determination of hormonal content of extracts.*

a) *Gonadotrophin.*

For titration purposes we employed 26—28 days' old infantile female rats of Løvens kemiske Fabrik stock. The dosage was spread over 5 subcutaneous injections in the course of 48 hours. Three animals for each dose were employed. The animals were killed on the fifth day, whereafter each uterus was isolated and weighed. The quantity of gonadotrophin producing an average tripling of the weight of the uterus with its fluid content was reckoned as one rat unit (R. U.).

Titration was performed by injecting a series of doses (0.5 — 1.0 — 2.0 — 3.0 — 4.0 — 6.0 — 9.0 ml.) of the urine extract. The gonadotrophin content in the extract is found in the usual manner by means of a dose-response curve.

In order to check the accuracy of the results, 10 days' urine from the same woman in the menopause was divided into 10 equal portions, whereupon 10 gonadotrophin analyses were made. The average value found was 40.2 R. U. per day, with a deviation of 12.2.

By means of this technique we are able to demonstrate down to 5 R. U. per day in the urine. At first, however, we

followed the technique described by *Thomsen & Pedersen-Bjergaard* (1936), in which, instead of the uterine weight, judgment is based upon the anatomical picture of the vaginal mucous membrane of the infantile rats. Vaginal epithelium being more sensitive than the uterine weight to the oestrin mobilized by the gonadotrophin, the microscopic examination of the vaginal epithelium makes it possible to show down to $\frac{1}{5}$ R. U. gonadotrophin, corresponding to 1 R. U. per day in the urine.

b) *Androgenic substances.*

The value here was determined according to *Jensen, Pedersen-Bjergaard & Tønnesen* (1944), a method in which the biological test is made on capons by application to the comb. One capon unit (C. U.) is reckoned as 400 x the quantity of hormone which, administered in the course of four days, on the fifth shows an average increase of 20 % of the comb area, which very closely agrees with the quantity of hormone which, injected intramuscularly twice daily in the course of four days, on the fifth shows a corresponding increase of the comb area.

For titration we employ adult, castrated cocks, which receive 0.05 ml. of the aforesaid alcoholic extract direct on the comb once daily for four successive days, whereupon the comb area is measured on the fifth.

Next, another set of capons is given 0.05 ml. of a suitable dilution of the alcoholic urine extract on the comb once daily for four successive days, the comb area being measured on the fifth.

On an average we employ 10 capons per analysis, or 5 per dose. The content of androgenic substance in the extract is ascertained by means of a dose-response curve.

For the purpose of gaining an impression of the accuracy of the results arrived at, ten days' urine from the same individual was divided into ten equal portions and each portion analysed for androgenic substance. The average value found was 5.43 C. U. per day with a deviation of 2.56.

c) *Oestrogenic substances.*

For titration we employed adult spayed mice of Løvens kemiske Fabrik stock. The dose was divided over three subcutaneous injections in the course of 48 hours. Three — sometimes six — animals were used per dose. The vaginal smears were examined 72, 80 and 96 hours after the first injection. One mouse unit (M. U.) is the amount of oestrin producing complete cornification of the vaginal smear in 60—70 % of the animals. Titration was performed by administering a series of dilutions according to the scheme described by *Kemp & Pedersen-Bjergaard* (1933). The oestrin content in the extract was found in the usual manner by means of a dose-response curve.

The accuracy of the results was checked by dividing ten days' urine from the same individual into 10 equal portions and making 10 analyses for oestrogenic substance. The average value found was 75 M. U. per day, deviation 18, using six animals to each dose.

MATERIAL

The investigation comprises the measured output from 360 individuals aged from 3 to 79 years. Only one or some few analyses for gonadotrophic and oestrogenic substances were made for each individual, in all 672 single determinations of each of the two hormones were made. Androgen analyses were not made with the same frequency as the other two, the total being 271 determinations.

The urine came partly from healthy, able-bodied individuals, and partly from patients not suffering from any endocrine disease.

RESULTS

1) *Oestrogenic substances.*

Fig. 1 shows the output of oestrogenic substance by normal females aged from 3 to 79 years. In all 672 values are plotted representing 360 individuals. Each value is given in the form of a small black dot. The thick curve — average curve — indicates the mean value appearing as the average of three-year

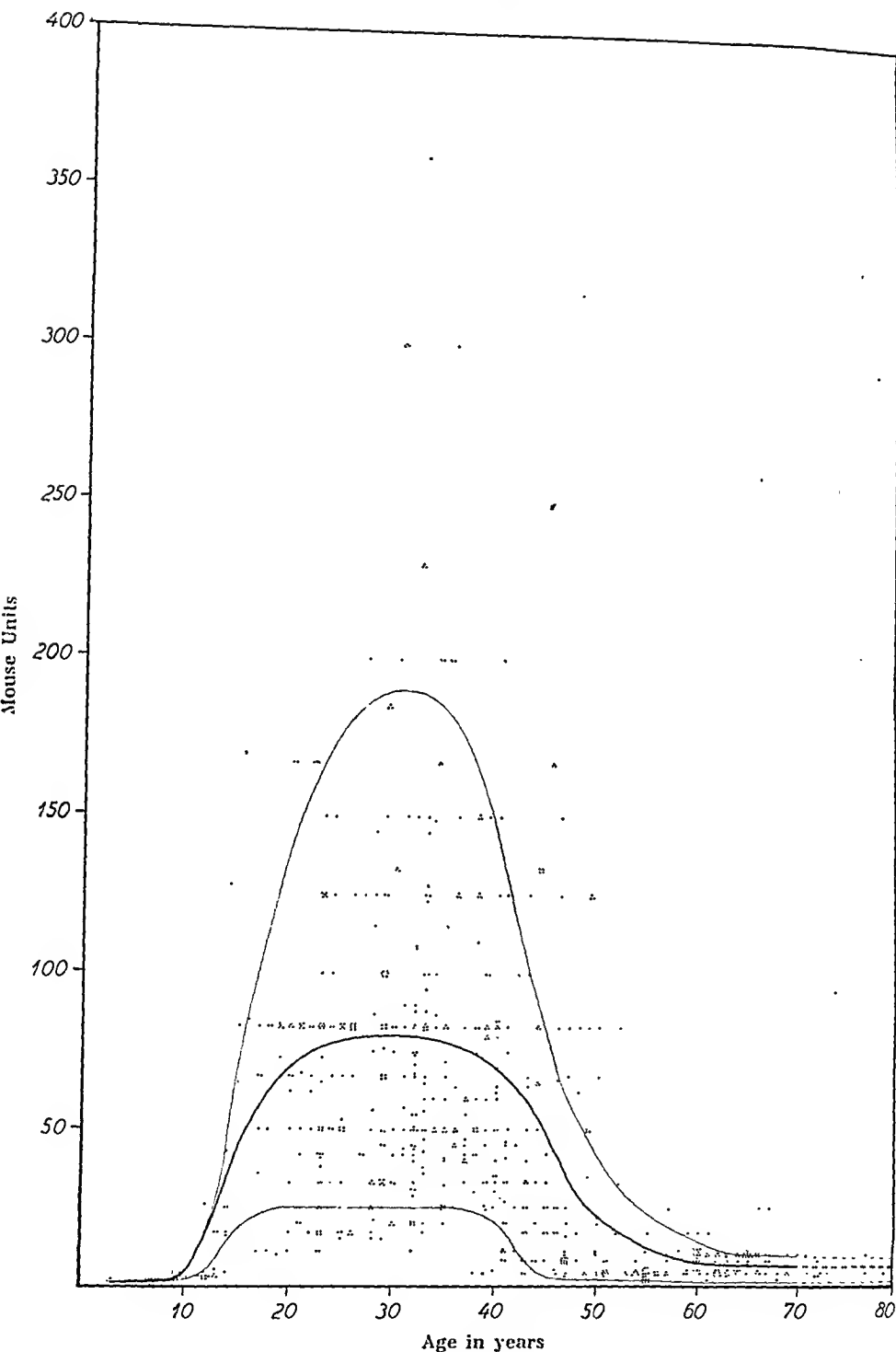


Fig. 1.

Output of oestrogenic substance in the urine of normal girls and adult women, expressed in mouse units per day. The curves are plotted through the average values for three years. The thick curve indicates the mean value. The two thin curves indicate the limits inside which 90 % of all values are placed.

periods. These triennial periods average 28 measurements each; the material, however, is somewhat heterogeneous in its distribution, most measurements being taken between the ages of 20 and 50 years, fewest for the youngest and oldest individuals. From 3 to 12 years the oestrin output is quite minimal, amounting up to 3 M.U. in the 24 hours; this agrees well with earlier works where, apart from the first post-natal days, in which *Neumann & Peter* (1932) found oestrogenic substances in the organism of the newly born, there are reports of only insignificant outputs of oestrogenic substances in the urine of children (*Dorfman, Greulich & Solomon* (1937)). *Nathanson, Towne & Aub* (1941) observed faintly increasing output $1\frac{1}{2}$ years before the menarche and incipient cyclic fluctuations in the output. *Dorfman, Greulich*

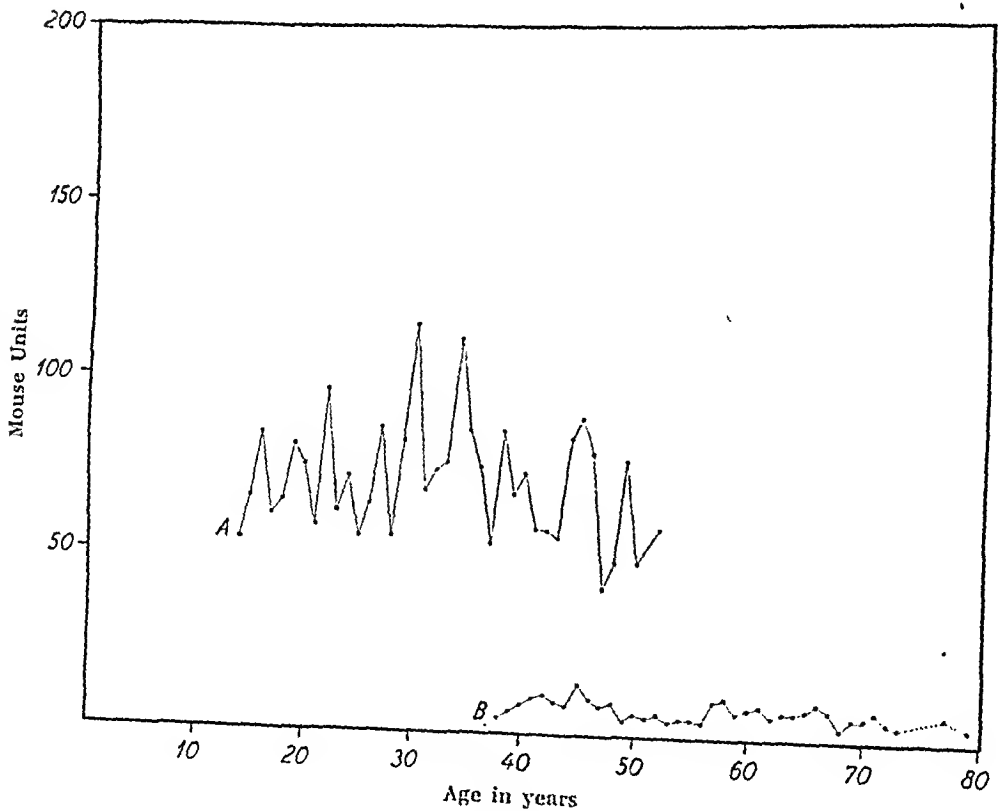


Fig. 2.

Curve A: Oestrin output of normally menstruating women.

Curve B: Oestrin output of post-menopausal women.

The curves are plotted through the average values for each year.

& Solomon (1937), like Hamburger (1938), pointed out as far as boys are concerned that the output of children in puberty is not proportional with age but with the development of the sexual characters. From the age of 12 (fig. 1) there is a steep increase in the output, which thereafter remains at the maximum between the 20th and the 40th year, when it approximates between 70 and 80 M. U. per day.

From the age of 40 the output of oestrin falls until about the age of 60 it has reached a value of 8 M. U., from which level it declines slowly towards lower values.

Whereas the curve in fig. 1 — »the life curve« for oestrin — already begins to descend steeply after the age of 40 because some of the individuals tested have become climacterial, the upper curve in fig. 2, representing only the normally menstruating females aged from 14 to 52 years, shows a more constant level.

The lower curve in fig. 2 represents in a similar manner the average annual values for women in and after the menopause. A characteristic feature of these two curves is their totally different levels. As long as the woman was menstruating, the average right up to the age of 50 years lay over 50 M. U., whereas for the menopausal women the value lies at 10 or less.

2) Gonadotrophin.

Whereas the output of oestrin increases suddenly when the menarche begins, the gonadotrophic hormone is mobilized slowly during growth, as fig. 3 shows.

Fig. 3 shows the output of gonadotrophin by normal females aged from 3 to 79 years. In all 672 values are recorded, representing 360 individuals. Each value is shown as a small dot. The continuous curve — »the life curve« for gonadotrophin — represents the mean value appearing as the average of three-year periods. These triennial periods comprise an average of 28 measurements each, but the material is not homogeneous in its distribution, as more tests were made between the ages of 20 and 50, less for the youngest and oldest individuals. From the age of 3 to 12 years the average output

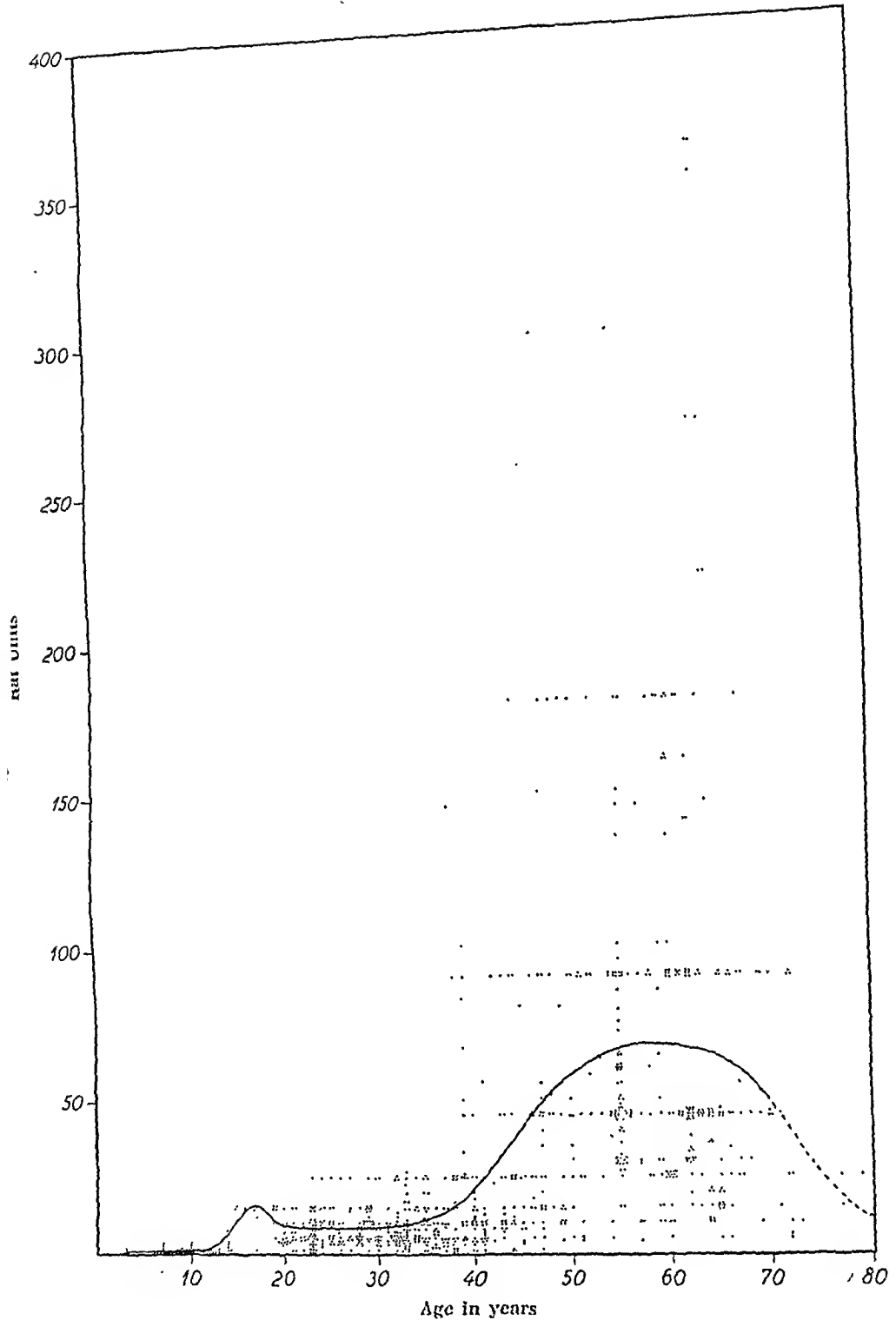


Fig. 3.

Output of gonadotrophic substance in the urine of normal girls and adult women expressed in R.U. per day. The curves are plotted through the average values for three years.

of gonadotrophin is very slight, amounting to one rat unit or less per day; from the age of 12, however, the output rises. This increase must undoubtedly be taken as an expression of an especially lively action of the pituitary gland in the years when genital development begins (*Hamburger (1947)*) and in fact has also been observed in boys (*Hamburger & Halvorsen (1942)*). At the age of 16 the curve reaches its first maximum at an output of 15 R. U. per day. After a fall to 9 R. U. per day around the age of 20 it remains at this level until the middle of the thirties, when the output again rises and continues to do so until the end of the sixties, when it reaches its second maximum at a value of 65 R. U. per day. After 70 years there seems to be another fall; but as the material here is sparse, we would not venture to conclude that this fall is significant.

Whereas the curve in fig. 3 from the age of 38 to 52 includes females before, during and after the menopause, in

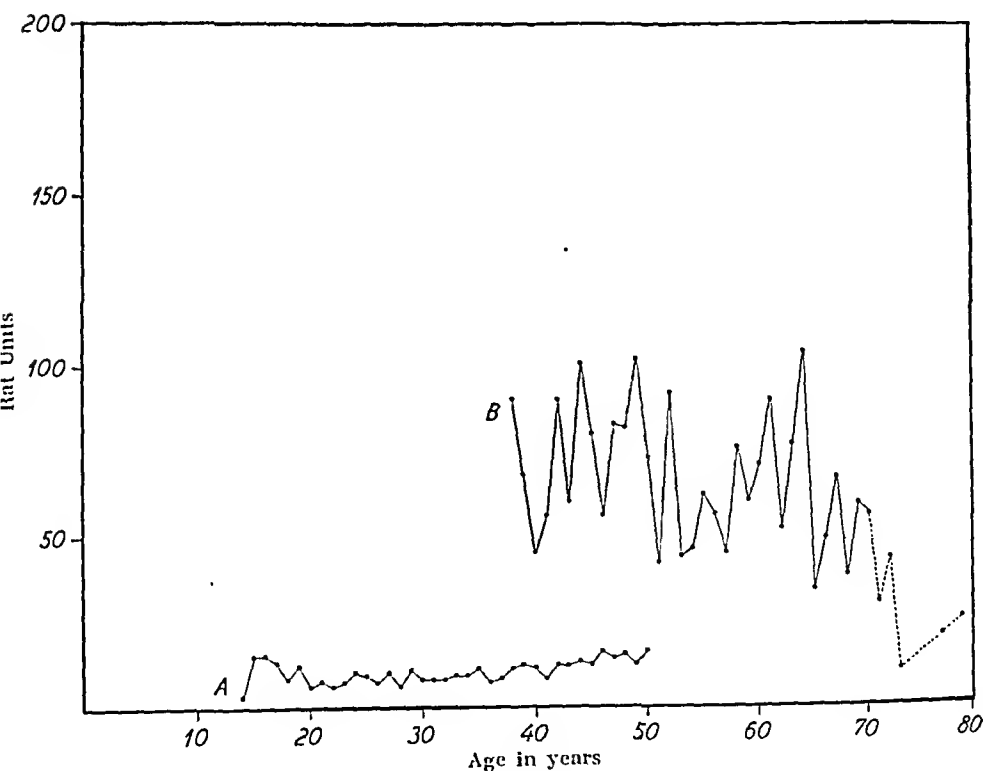


Fig. 4.

Curve A: Gonadotrophin output of normally menstruating women.

Curve B: Gonadotrophin output of post-menopausal women.

The curves are plotted through the average values for each year.

fig. 4 we have divided the material into normally menstruating and post-climacterial women.

As in the case of fig. 2, which shows the corresponding curves for oestrin, one observes the totally different levels of the curves for women before and after the menopause. But, in contrast to the curves in fig. 2, the curve for the normally menstruating women in fig. 4 is the lower one, and that for the menopausal women the upper.

The explanation is that the oestrin output is reduced when the ovarian function ceases; at the same time there is a cessation of the regulating influence of oestrin upon the pituitary gland, whose output of gonadotrophins rises to a higher level, where it remains until the death of the individual. In the first two graphs in fig. 5 we see the output of two post-climacterial women. It should be interposed here, however, that the gonadotrophin output of post-climacterial women may periodically amount to low values; the normal, however, is an increased output (30 to 180 R. U. per day) in comparison with that of menstruating women, for whom the highest value measured is 25 R. U. per day.

Among the first communications on increased gonadotrophin output by women after the menopause in relation to that of normally menstruating women were those of *Zondek* (1930 I), who found an increase in 25 %, *Oesterreicher* (1932) in 90 %, and *Sæthre* (1933) in 92 %. Later on *Zondek* (1935) succeeded in finding increased output in 58 %, and *Oesterreicher* (1933) was actually able to demonstrate the increased output in all individuals, provided a sufficient number of analyses were made from each — after the menopause the same individual sometimes excreted the same amount of gonadotrophin, sometimes higher quantities in relation to normally menstruating women. We have determined an increased output of gonadotrophin in 84 % of the post-menopausal women examined in this paper.

In the case of a number of preclimacterial women we have been able to observe outputs of greatly varying quantities, fluctuating from the normal excretion (oestrin and gonadotrophin) to polyfolliculin excretion (high oestrin — normal

gonadotrophin) and polygonadotrophin excretion (normal oestrin — high gonadotrophin). It was seen that the various phases fluctuated most arbitrarily.

One example of the fluctuations in a single individual will be seen in the last graph, fig. 5, in which in four successive months we made one determination of gonadotrophin and oestrin once a month.

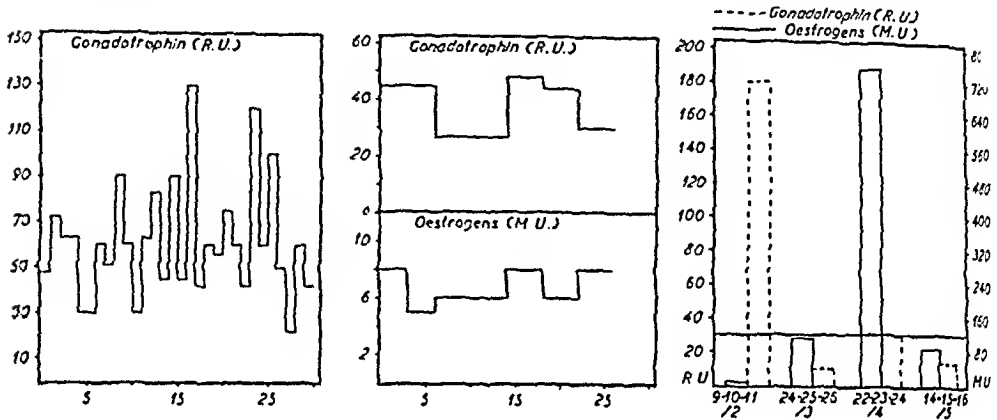


Fig. 5.

Graph 1. Daily output of gonadotrophic hormone in the urine of a post-menopausal 58 year old woman.

Graph 2. Output of gonadotrophic hormone and oestrin in the urine of a post-menopausal 55 year old woman.

Graph 3. Output of gonadotrophic hormone and oestrin in the urine of a preclimacteric 47 year old woman.

Abseissa: Days.

Ordinate: Oestrin and gonadotrophin output in M. U. and R. U.

It will be seen from Graph 3 of fig. 5 that the four analyses show: February: increased gonadotrophin excretion.

March: normal excretion.

April: increased oestrin excretion.

May: normal excretion.

On a previous occasion we have measured the output of gonadotrophic hormone and oestrogenic substances a whole month for 11 women. The results are shown in the diagrams in fig. 6.

A feature common to the sets of curves in fig. 6 is the low output of oestrogenic substances in the menstruation period.

In the intermenstrual phase there is an increase, reaching its maximum on the 10th—11th—12th day of the cycle, followed by a fall in the middle of the cycle whereupon there appears a secondary increase with a maximum output on the 22nd—23rd and 24th day of the cycle. The output amounts to between 20 and 200 M. U. in the 24 hours. Most workers agree that the first increase in the oestrin titre is recorded on about the 10th—14th day of the cycle, there is considerable variation in the records of the secondary increase, which some place to the 19th—21st day, others to the 25th—26th day (Siebke (1930), *Gustavson & Greene* (1934), *Frank* (1935), *Pedersen-Bjergaard* (1936), *Palmer* (1937), *Gustavson, Mason, Hays, Wood & D'Amour* (1938), *Smith, Smith & Pincus* (1938), *Lackner, Wachtel & Soskin* (1938), *von Haam & Rothermich* (1940), *Furuhjelm* (1940), *Dingemanse & Laqueur* (1940) and *Genell* (1943)).

The output of gonadotrophin amounts to between 1 and 25 R. U. per 24 hours. No systematic fluctuation has been observed as in the case of oestrin.

In one instance we had occasion to find an oestrin output which, as distinct from the normal, reached its maximum in the very days of menstruation, whereas it was low in the intermenstrual days. This being a matter of particular interest we shall give a brief account of the case report of the individual; the full report was given by *Hermann & Schroder* (1935).

An unmarried domestic servant, 35 years old, admitted to the Psychiatric Department of the Municipal Hospital, Copen-

Text to fig. 6.

Diagram	Age in years	Length of cycle in days	Length of menstruation in days
a	23	28	4
b	28	28	4
c	32	28	3—4
d	32	29	3
e	33	27	3
f	33	31	4
g	33	27	3
h	33	26	5
i	38	25	4

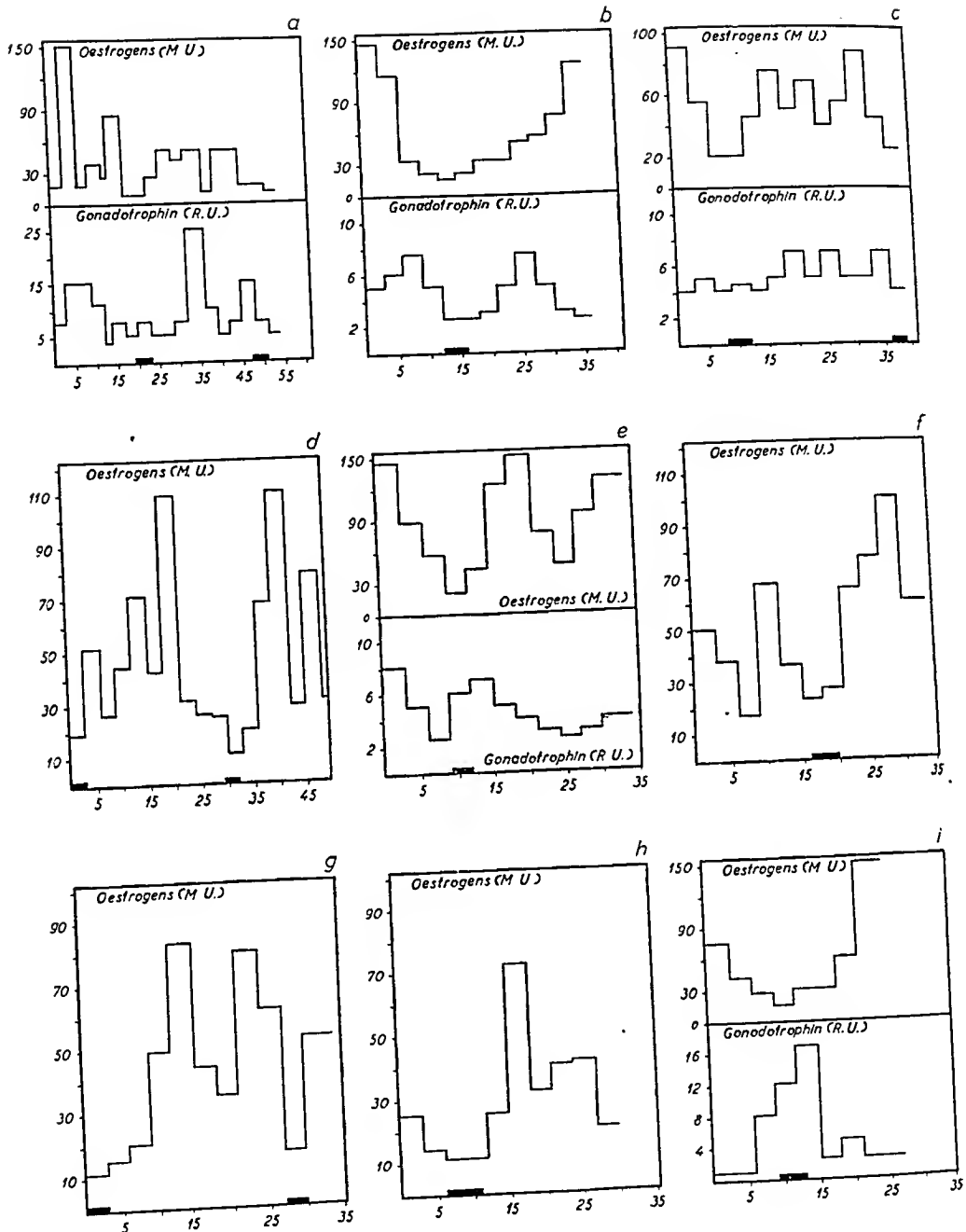


Fig. 6.

Output of oestrogenic and gonadotrophic substances in the urine of 9 normal women.

Abscissa: Days.

Ordinate: Output of oestrin and gonadotrophin in M.U. and R.U.

Each step represents the average of the output of three days.

hagen, for psychopathia, epilepsia antea and exhibitionism. Menses irregular since the age of 14. States that in her menstruation periods she is seized with an irresistible want to exhibit and masturbate publicly. Her oestrin output is shown in fig 7.

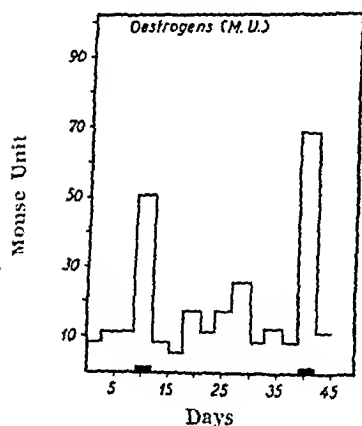


Fig. 7.

Output of oestrin in the urine of a 35 year old female patient with want of exhibition during menstruation. (Each step represents the average of three days' output).

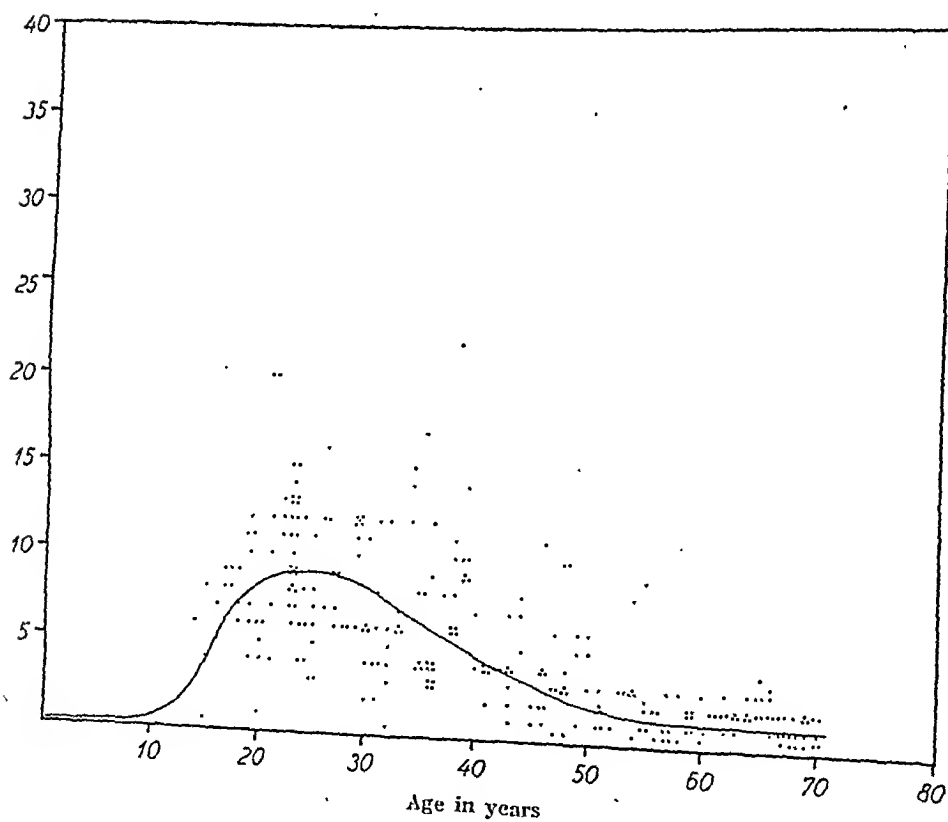


Fig. 8.

Output of androgenic substances in the urine of normal girls and adult women expressed in capon units per day. The curve is plotted through the average values for three years.

3) *Androgenic substances.*

The output of androgenic substances is illustrated by fig. 8.

It will be seen that with very few exceptions the output is between 4 and 25 C. U. in the 24 hours for females between the ages of 20 and 40. The average curve shown in fig. 8 is very similar to that published by *Hamburger, Halvorsen & Pedersen* (1945).

4) *Hormone analysis in blood.*

The desire has frequently been expressed for a more direct expression of the hormone picture of the blood by analyzing the blood for hormones instead of the urine. With present-day biological methods, however, it is impossible with constancy to demonstrate oestrogenic, androgenic and gonadotrophic substances in the blood, because the concentrations are so low that a direct injection of the blood as a rule fails to give a positive result. Preparing an extract from a large quantity of blood encounters the difficulty in practice that blood, compared with urine, can be procured only in limited volume.

The blood from 20 normal women aged 20 to 40 years proved in 5 cases to contain 25 to 50 R. U. gonadotrophic hormone per litre, whereas it was impossible to demonstrate the hormone in the other 16 cases because the concentration was too low. The same can be said of the oestrogenic and androgenic substances. Another thing is that the value of a blood analysis has its limits, because it indicates the concentration in the blood only at the time of taking the sample; it tells nothing of the size of the production within a certain period as for example the urine analysis, which expresses the total output over 24 hours.

5) *Oestrogenic substances in the urine from ovariectomized women.*

The oestrin found in the urine of normally menstruating women comes partly from the ovaries and partly, in smaller quantities, from the adrenals. The fact that the share of the adrenals in the amount of oestrin produced is very small

appears from the low oestrogen output of young ovariectomized women. For 26 of these we find an output averaging 8 M. U. per day, corresponding to 224 M. U. in a four week period, which again is less than 15 % of the oestrin excreted by normally menstruating women in the course of a cycle.

DISCUSSION

1) *Correlation between hormone output and age.*

The *gonadotrophic hormone* is mobilized slowly as the years advance. Up to puberty the excretion is slight, amounting at the most to some few rat units; from the twelfth year it increases and by the sixteenth year has reached about 15 R. U. per day. Thereafter the excretion decreases to about 9 R. U. until the beginning of the menopause at the age of forty. From about the thirty-eighth year to the seventieth the output rises quite steadily to 65 R. U. per day. After the seventieth year, however, there seems to be a downward trend in the output.

Prior to puberty the *oestrogenic substances* occur in very small quantities in the urine, at most a few M. U. per day; but from the twelfth year, at the inception of puberty, the excretion rises suddenly and until the menopause at the age of 40 to 50 has values between 17 and 200 M. U. per day. After the menopause the output falls to less than 20 M. U. per day, from which level it decreases slowly in senility.

The *androgenic substances* before puberty are found in very small quantities, up to one or two capon units per day; but after puberty has begun the output of androgen in the urine rises to 8. C. U. at about the thirtieth year. Thereafter it decreases steadily to 1 C. U. per day during the subsequent decades.

2) *Correlation between gonadotrophin and oestrin outputs.*

On comparing the two »life curves« for gonadotrophin and oestrin it will be found that up to puberty the excretion of both hormones is very small, but that thereafter the gonadotrophin output increases relatively more than that of oestrin. This preponderance, however, lasts only for about three years, and then

the gonadotrophin output falls some few units, whereas the oestrin output continues to increase and reaches the maximum between the ages of 20 and 40. At this stage the oestrin output falls abruptly again at the same time as the quantity of gonadotrophin in the urine begins to increase. Whereas oestrin continues to fall with advancing age, the gonadotrophin output steadily increases to the age of seventy. It is only at this relatively high age that the general retrogression of the activity of the life functions also influences the production of gonadotrophin. It would thus seem that there is a certain correlation between the gonadotrophin and oestrin outputs, the latter being high when the former is low, and vice versa. This does not apply before puberty, when both values are low; but nor does it always apply in the menopause, when we sometimes find an increased excretion of gonadotrophin, for example one about 45 R. U. and a normal oestrin excretion of 50 M. U. Such cases are relatively rare, however, and the abnormal excretion ceases in the post-menopausal stage.

3) *The cyclic fluctuations.*

Within one and the same menstrual cycle the lowest output of oestrogenic substance is observed in the menstruation period, about 20 M. U. per 24 hours. In the intermenstrual phase there is an increase in the output, one that reaches its maximum on the 10th, 11th and 12th cycle days, followed by a slight fall in the middle of the cycle, whereupon there is a secondary rise with a maximum output on the 22nd, 23rd and 24th days. The high outputs in the intermenstrual period amount to between 100 and 200 M. U. As regards the excretion of gonadotrophin, no systematic rhythmic fluctuation corresponding to the oestrin excretion is observable within a cycle. The correlation between gonadotrophin and oestrin as visualized by the two »life curves« is not observable within a single cycle.

4) *Determination of output limits.*

The numerical results of our investigations are summarized below.

Gonadotrophin:

From the 3rd to the 12th year the output amounts up to some few rat units, with a maximum of 3 R. U. for normal girls.

From the 12th to 38th year we have found values from 1 R. U. to 15 R. U.

From the 38th year and onwards we have found outputs of from 5 to 360 R. U.

Oestrogenic substances.

From the age of 3 to 12 the output is quite low, at most 3 M. U. per day.

From the 12th to the 15th year we have observed up to 128 M. U. per day.

From the 15th to the 38th year the output varies from 17 right up to 360 M. U., usually between 20 and 200 M. U.

After the 38th to the 52nd year we have found values from 8 to 200 M. U. per day.

After the 52nd year always less than 25 M. U. per day.

Androgenic substances:

Up to the 12th year we have found only very small values, up to two capon units per day.

After puberty the output increases steeply and reaches its maximum at 30 years with values from 1 to 22 C. U. per day.

Thereafter the output decreases steadily until at about the 60th year when it again is some few C. U. per day.

SUMMARY

The purpose of this investigation was to examine the excretion of oestrogenic, androgenic and gonadotrophic substances in the urine of normal women in all age groups, in order to make a material available as a basis for the evaluation of clinical analyses from patients. We aimed at elucidating the average output and also the normal physiological margin of variation in the output of these substances.

The material comprises 360 normal women aged from 3 to 79 years.

Gonadotrophin was assayed in infantile female rats by the tannic acid precipitation method. Oestrogenic and androgenic substances were determined after combined acid hydrolysis and carbon tetrachloride extraction in spayed mice and on capons.

Gonadotrophin.

Until puberty the quantity of gonadotrophin excreted is scarcely demonstrable. For normally menstruating women the quantity is from 0 to 25 R. U. per 24 hours, with a slight increase during pubescence and a more marked one after the menopause, when the output may amount from 5 to 360 R. U. per day.

Oestrogenic substances.

Until puberty there are scarcely demonstrable amounts of oestrin. Normally menstruating women have a 24-hour output of from 20 to 400 M. U., with a fall after the menopause, when the output is never more than 25 M. U. The lowest values are encountered during menstruation, but on the whole the output is subject to uncharacteristic diurnal variations with a trend towards a maximum in the middle of the intermenstrual period, or sometimes towards two maxima in this period.

Androgenic substances.

The excretion gradually increases from puberty and reaches a maximum at about the age of 30, followed by falling values.

As the practical consequence of these investigations it is possible to draw the following conclusions as to when the hormone excretion may be regarded as pathological or at any rate remarkable:

1. > 30 R. U. gonadotrophic hormone for women before the menopause.
2. < 20 M. U. and > 400 M. U. oestrogenic hormone in the intermenstrual period.

3. > 25 M. U. oestrogenic hormone for women after the menopause.
4. > 25 C. U. androgenic hormone.

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PITUITARY HYPERTENSION

BY

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The notion of pituitary hypertension does not exist in ordinary medical and endocrinological text-books. But occurrence of blood pressure disturbances in association with pituitary diseases is a well-known phenomenon. Lowered blood pressure values are the most common, such being characteristic of the different syndromes due to pituitary insufficiency, in particular Simmonds' syndrome, but also of pituitary adiposity (*Goldzieher*, 1939, p. 107), and occasionally also of adiposogenital dystrophy. Personally I have ascertained a lowered blood pressure in association with different diseases due probably to insufficiency of the pituitary-hypothalamic system. *Goldzieher* (1939, p. 389) points out that particularly postural hypotension is characteristic. He even regards this symptom as pathognomonic of pituitary insufficiency.

We should then expect hypertension to be a common occurrence in association with conditions due to pituitary hyperfunction (e. g. gigantism and acromegaly). However, the literature does not bear out this hypothesis. Hypertension as a symptom of acromegaly is mentioned, apart from a couple of casuistic reports, by few writers only, e. g. *Kyllin* (1943). Cushing's syndrome, on the other hand, is known to be characterized among others by hypertension. This syndrome was

originally supposed to be due to a basophilic adenoma in the pituitary body, but is now believed to have a more complicated pathogenesis, where the adrenal cortex plays a prominent part.

To my knowledge *Kylin* is the only writer to have attached any particular importance to the pituitary body in the pathogenesis of hypertension. *Kylin* suggests a »pituitary form of essential hypertension«, which he believes to be caused by an ordinary hyperfunction of the pituitary body, but probably in particular of the basophiles in the anterior pituitary lobe.

The connexion between pituitary body and a raised blood pressure being still very uncertain, I think there is reason to report the following case history of a patient suffering from hypertension, on the pituitary origin of which there can hardly be any doubt.

CASE HISTORY

Frederiksberg Hospital, Neurological Dept., Case 367/46, married woman, aged 44.

No known predisposition.

The patient has since childhood been suffering from headache, though not very severe. In addition she was always somewhat nervous, easy to frighten, and possibly a little backward at school. Menses from the age of 15, scanty but regular. One parturition (delivery by forceps) in 1927, followed by infection with confinement to bed for 3 months.

The patient's *present disease* commenced in 1942 together with the occurrence of longer intervals between the menstrual bleedings. Since 1942 only few scanty bleedings, once in 1944, and twice in 1945, the last in August. The disease manifested itself by increased headache, dizziness, everything going black, fatigue, hot flushes, and perspiration; furthermore by pains in back and precordium, shivering fits, and intermittent functional dyspnoea. The disease as a whole was steadily progressive, but with characteristic paroxysms, particularly of the cardiac symptoms. There occurred some impairment of me-

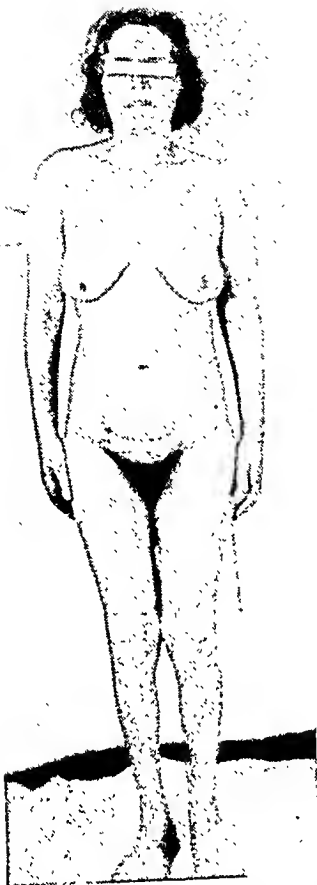
mory, and the patient became somewhat depressed, because, on account of her disease, she could do no work whatever from the end of 1944. Her symptoms were first diagnosed as menopausal, and, because of the mental symptoms, the patient was in August 1944 admitted to a psychiatric department, where cardiac affection was ascertained. She was, therefore, transferred to a medical department, where heart disease (myocardial degeneration, angina pectoris) and arteriosclerosis with hypertension were diagnosed. The blood pressure was then 180/140.

The patient was admitted to our department in November 1946. We were then further informed that the patient's feet had increased somewhat in size ab. 10 years previously, and that a light facial hypertrichosis had developed about 5 years previously, while at the same time the hair of her head had become bristly. Within the last year before her admission to this department her hands had become swollen and red, while rhagades had occurred in the corners of the mouth, as well as a smarting and burning pain in the tongue.

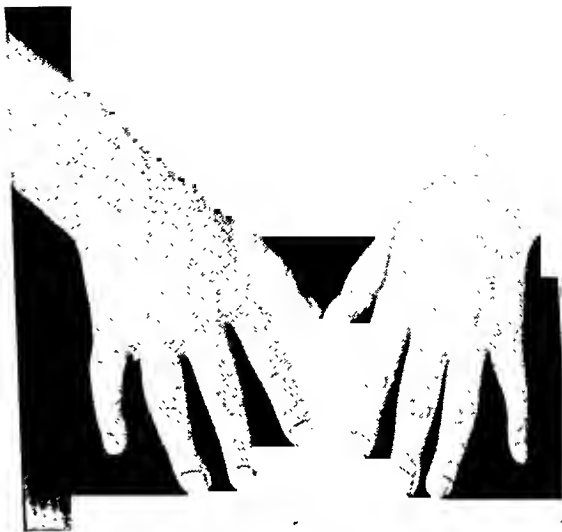
Physical examination on admission:

Psyche: Indolent, somewhat mentally defective.

Appearance: Tired and poorly, with inconsiderable mimics. Nails brittle and slightly domed. Appearance on the whole slightly acromegalic: a somewhat domed forehead with projecting supereiliary margins. Nose rather big (pictures 1 and 2). Her tongue is red with large furrows. Rhagades in the corners of the mouth. Her hands are large, clumsy, and succulent, with a glossy skin (picture 3). The skin is pallid and in the general dry. The growth of hair on her head is greyish, thick but dry and bristly. Excessive hairiness in the face with a number of long hairs on upper lip and chin. Light growing together of the eyebrows. A few hairs on the sternum. Axillary hairs normal. Pubes thick, superiorly with an almost virile delimitation, but with no hairs along the linea alba. Laterally the pubes are continuous with a thick, ab. 1 cm. long femoral growth of hair vanishing at the knees (picture 4). No crural hairiness, but a rather fine growth of hair on the forearms.



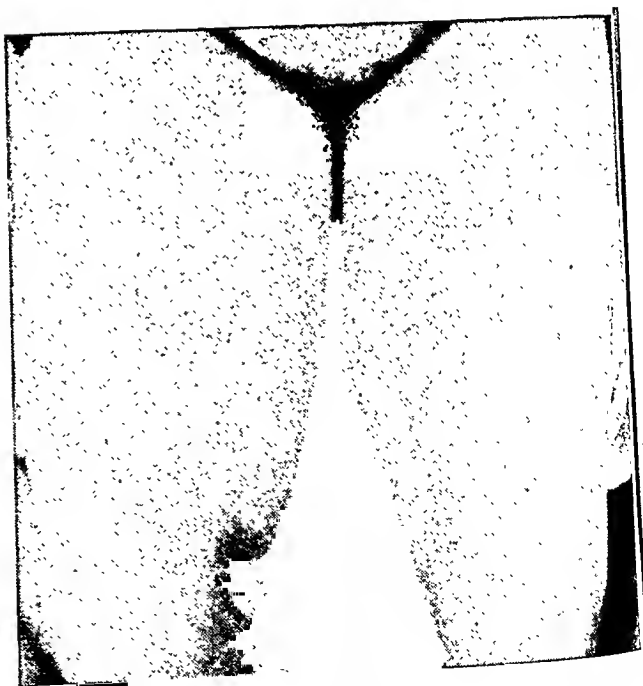
Picture 1.



Picture 3.



Picture 2.



Picture 4.

Neurological examination: Normally active deep reflexes in upper extremities. In lower extremities hyperactive deep reflexes with foot clonus, moderate increase in muscular tension, but atrophic muscles. Gait somewhat uncertain, and a tendency to fall at Romberg's test. Otherwise the neurological examination revealed nothing abnormal.

Ophthalmoscopy, carried out several times, revealed almost the same conditions throughout: Narrow arteries differing in caliber, winding veins, pronounced Gunn's phenomenon, numerous scattered haemorrhages and exudates.

Spinal fluid (14/11/45): $\frac{2}{3}$ cells, globulin 1—2, albumin 25—30. W. r. neg. (15/4/46): $\frac{5}{3}$, 0—1, 14—15.

X-ray of skull: Hyperostosis frontalis int., l. gr., sella normal. Stethoscopy of heart: Nothing abnormal. X-ray of heart: Slight enlargement. Ecg.: T_1 and T_2 iso-electric. S- T_1 & 2 slightly depressed (degeneratio myocardii l. gr.).

The renal examinations revealed no signs of renal disease. Urine analysis, Addis' concentration test, blood urea, standard clearance, and urograms: Normal conditions.

X-ray examination with a view to adrenal conditions showed nothing abnormal (pneumography not undertaken).

Blood examination: Leucocytosis (14.840—9.440); otherwise normal conditions. Fractional determination of cholesterol and protein in serum showed normal conditions. Ewald's test meal: Nothing abnormal.

Hormone analysis on urine showed figures corresponding to those of the woman's postclimacteric age (45 R. U. of gonadotrophine; less than 8 M. U. of oestrogen; and 2 C. U. of androgen).

Glucose tolerance test (both before and after X-rays on the pituitary body) revealed a normal fasting blood sugar, but an abnormally high rise, up to 220 mg. %.

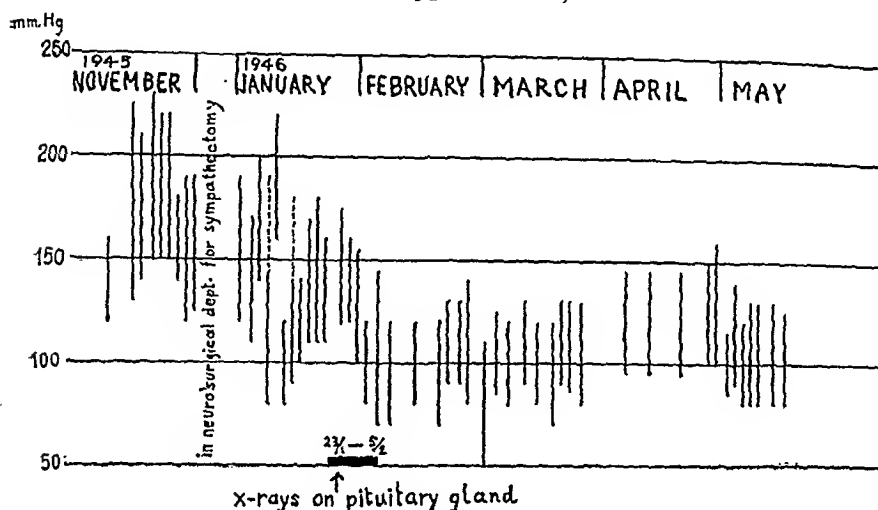
The basal metabolic rate was considerably increased before the X-ray treatment (145—138—150 %), but fell slowly to normal values after the treatment (121—118—107—100 %).

The blood pressure was always increased, but rather fluctuating, before the X-ray treatment (see Diagram).

Height 168 cm. Weight 69.5—59.3 kg.

Diagram illustrating the patient's blood pressure (systolic and diastolic) from Nov. 9, 1945 to May 17, 1946.

The dotted lines indicate an abrupt rise in blood pressure (paroxysmal hypertension).



Course: From Dec. 4, 1945 to Jan. 2, 1946 the patient was in the Neurosurgical Dept. of the *Rigshospital*, where, on account of the hypertension, bilateral sympathectomy was done according to *Smithwick & White*. The operation had, however, a transitory effect only on the blood pressure, and no effect whatever on the other symptoms.

While staying in *Frederiksberg Hospital* the patient had several attacks of paroxysmal hypertension, during which she was feeling very poorly with a sensation of pressure in the chest and nausea. During the attacks she was pale, cyanotic and sweating with a fast and soft pulse. Very occasionally a highly irregular heart action was ascertained. The blood pressure rose once during such an attack from 145/80 to 190/120, and during another attack to 220/150. These attacks, generally lasting up to half an hour, were almost more pronounced after the sympathectomy. Between the 20th and 21st of January the patient developed within a few hours a *left* central facial paresis and left hemiparesis, but without loss of consciousness.

The patient's disease being supposed to be of pituitary origin, X-ray treatment of the pituitary body was commenced

on Jan. 23, 1946. A total of 800 r, distributed over 2 fields, were given till Febr. 5. The X-rays had an excellent effect. The blood pressure, which before the treatment had been permanently increased, both the systolic and the diastolic, though rather fluctuating, began to fall already after a few treatments, to become perfectly normal before the conclusion of the course of treatment. It remained normal and far more stable (see Diagram) during the rest of her stay in hospital ($3\frac{1}{2}$ months). The basal metabolic rate likewise fall to the normal, and the patient felt far better both mentally and somatically. The previous attacks of paroxysmal hypertension occurred no more. The headache and the dizziness vanished completely. The hemiparesis had decreased so much by the time the patient was discharged that she was able to manage alone with one stick. Her temper was far more stable, and she was more interested and far less indolent than previously. She was discharged from the department on May 18, 1946.

The improvement did not last, however. A few months after her discharge she again developed increasing headache. On Sept. 1, 1946 the patient was admitted to the psychiatric department with barbituric acid poisoning, after having eaten too many dormitives in order to quiet down. The blood pressure was then again considerably increased (240/155—190/120), but the patient presented no fresh signs or symptoms. A symptomatic treatment was now attempted for the patient's hypertension. In the surgical department an anastomosis was made between the radial artery and a subcutaneous vein in the *tabatière* on one side. However, this operation had no definite blood-pressure-reducing effect.

EPICRISIS AND DISCUSSION

The patient is a woman, aged 44, whose disease commenced 4 years previous to her admission to this department. It came on with menostasis, after which the patient developed increasing headache, dizziness, tiredness, hot flushes, and perspiration, as well as steadily progressing, yet intermittent dyspnoea. The patient being quite naturally in low spirits she

was treated first in the psychiatric department, after which she was transferred to the medical department on account of heart disease and hypertension. Was treated for these affections through 2 years, until she was admitted to this department. Here we were informed that the patient's appearance had changed slightly in the acromegalic direction within the past 5 to 10 years. Physically the patient revealed pronounced hypertension, but with considerable fluctuations and proper paroxysms. There were no signs of renal disease, but severe cerebral and cardiac symptoms had developed in consequence of the hypertension. In addition to the hypertension and its sequelae there was, however, found a clinically well-pronounced hyperpituitarism with hyperproduction of practically all the hormones of the anterior pituitary lobe. The patient had a slightly acromegalic appearance, indicative of an increase in the production of growth hormone, and a fairly pronounced hypertrichosis, probably caused by an increase in the corticotrophic hormone production. The basal metabolic rate was considerably raised, probably due to hyperproduction of the thyrotrophic hormone, and there were signs of pituitary diabetes with reduced carbohydrate tolerance, presumably on account of an increased diabetogenic hormone production.

Apart from the hypertension and its sequelae all the patient's symptoms can thus be explained as due to a hyperfunction of the anterior pituitary lobe. Hence it seems reasonable to presume that also the hypertension is released from the pituitary body on account of an increased hormone production. This is the more likely because in the present case the hypertension was not due to a renal disease, nor was it reminiscent of the usual essential hypertension, particularly on account of the marked lability of the blood pressure in our patient. Such lability should, it seems, be characteristic of hypertension caused by a hormonal disturbance. At least it is very characteristic of the hypertension due to tumours in the adrenal medulla or other pheochromocytomas, but is by *Kylin* pointed out as characteristic also of the pituitary hypertension described by him; and there were no signs of a pheochromocytoma in our patient.

The most conclusive evidence of the pituitary origin of the hypertension was, however, the striking effect of X-rays on the pituitary body. After this treatment the blood pressure remained perfectly normal through 5 or 6 months without greater fluctuations than in normal individuals, whereas neither sympathectomy nor arteriovenous anastomosis had a definite effect on the blood pressure. The fact that the blood pressure rose again about 6 months after the X-ray treatment does not, in my opinion, go against the theory of the pituitary origin of the hypertension, on the contrary it seems rather to argue in favour of this theory.

Thus, there seems hardly to be any doubt that the primary cause of the hypertension in our patient was a pituitary disturbance. The exact pathogenesis is, however, rather uncertain; but the pituitary disturbance consisted probably in a hyperproduction either of a special blood-pressure-regulating hormone (the existence of which is still hypothetical) or of the adrenotrophic hormone, so that the effect passed by the adrenal medulla (the pheochrome system).

I shall not enter further on the still very uncertain problem of the normal regulation of the blood pressure. Only there is reason just to mention that there is found interaction between the blood-pressure-regulating centres in the medulla oblongata and the central system, i. e. pituitary body, hypothalamus, and cerebral cortex (psyche) as well as the peripheral system, both through the autonomous nervous system (by the presso-receptor zones in the carotid sinus and elsewhere) and the hormonal system (by the pituitary body to the different endocrine glands, notably the adrenals). A disturbance anywhere in this regulating system may thus interfere with the blood pressure.

It seems a rather certain fact that hypotension — perhaps particularly the postural hypotension — is often due to pituitary insufficiency. And I feel convinced that many cases of »essential« hypertension will be found to be associated with a pituitary hyperfunction, if more attention will be given to this possibility.

SUMMARY

A case is reported of hypertension in a woman, aged 44, commenced 4 years previously, simultaneously with the menopause. Prior to the patient's admission to this department her disease had been diagnosed and treated as »essential« hypertension, but here the hypertension with its pronounced cardiac and cerebral complications was found to be attended by marked signs of anterior pituitary hyperfunction: 1) slightly acromegalic appearance (increased growth hormone production), 2) hypertrichosis (increased corticotrophic hormone production?), 3) considerable rise of basal metabolic rate (increased production of thyrotrophic hormone, or of a special hormone regulating the metabolism?), 4) light pituitary diabetes (increased production of »diabetogenic« hormone). The blood pressure is rather fluctuating with several attacks of »paroxysmal hypertension«, but there is no evidence to suggest the presence of pheochromocytoma. Thus, the entire clinical picture seems indicative that the hypertension is of pituitary origin (increased production of adrenotrophic hormone, or of a special blood-pressure-regulating hormone?). This hypothesis was borne out by the fact that X-ray treatment of the pituitary body had the effect that the blood pressure fell to normal values and remained normal and stable for 6 months.

»Essential« hypertension may perhaps be explained as due to anterior pituitary hyperfunction in no small number of cases.

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From the Pharmaco-therapeutic
Laboratory of the University of Amsterdam.

GROWTH STUDIES

(I. In rats and in human dwarfs)

BY

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assisted by A. Kemp and J. C. van Houten

INTRODUCTION

We began to test hypophyseal growth substances by weight increments produced in normal female »plateaued« rats, as described by those who pioneered in this field. We then changed over to a similar assay for which (younger) hypophysectomized male rats were used. They were sooner available in our colony and they offered the additional advantage of sensitivity, one or two weeks shorter experiments and consistent results, since young adult hypophyseoprivic rats gain no weight after hypophysectomy. This test was also taken over from earlier descriptions by other authors whose work was reviewed by H. B. van Dyke in *The Physiology and Pharmacology of the Pituitary Body*, Vol. I, 1936 and Vol. II, 1939. There was one technical modification added to the subject, when the hypophysectomy along the parapharyngeal route was simplified to the form in which we used it many thousand times (*Freud* (1938)¹).

The last ten years we definitely adhered to skeletal indicators of deficiency and substitution of the hypophyseal growth principle. Our decision was prompted by a survey of the ske-

leton in growing rats (*Freud & Levie (1938)*) and by the consistent observation, that hypophyseal extracts restored skeletal growth, when this was arrested by hypophysectomy. Our test method now consisted of hypophysectomy at 6 to 8 weeks of age of male rats, when their weight was rather less than 100 Gm.

As in such rats the changes of the epiphyseal cartilage discs are most marked among the responses of the skeleton, it was proposed to designate the principle producing this effect as chondrotrophin (*Freud (1938)*, *Freud, Levie & Kroon (1939)*, *Levie (1939)*). Chondrotrophin maintains or restores epiphyseal cartilage eutrophy and on this effect a short test was based by the Californian group of workers (*Evans et al. 1943*)).

Admittedly chondrotrophin expresses an arbitrary preference for one of several effects of the growth principle, as e. g. the word oestrogen expresses only a few effects produced by that class of substances. Nevertheless we prefer to speak of chondrotrophin rather than of somatotrophin (*Selye (1947)*), because if the first word expresses too little, the last one probably indicates more than what really is attributable to the growth principle.

We disagree with those authors who advocate other than skeletal indicators of chondrotrophin activity in non hypophysectomized animals. Even (hypopituitary) hereditary dwarf mice are not acceptable substitutes, though in these weight increment may be parallel with skeletal growth (*Fønns-Bech (1947)*). Difficulties were also created when hypophyseal extracts proved effective at the prevention of growth stasis and at growth promotion in normal and in hypophysectomized pigeons (*Miller & Riddle (1943)*). Our objection does not concern, of course, investigations of whatever character so long as no comparisons are made between doubtfully comparable things. No evidence has been advanced to show that chondrotrophin potency is being measured when another indicator is used than *cartilage eutrophy* in hypophysectomized mammals (preferably rats). Moreover skeletal growth may be handicap-

ped by deficiencies other than chondrotrophin and restored by providing substitution. Therefore it remains an unavoidable necessity to ascertain the part played by chondrotrophin in growth promotion. To achieve this, it is necessary to eliminate intrinsic hypophyseal agents from the experiment and to test the role of other than chondrotrophic fractions in the result obtained with unfractionated extracts (*Li et al. (1945)*, *Li (1947)*, *Marx et al. (1944)*).

It is subject to doubt that gain in body weight would be parallel to skeletal growth. It is certain that human adults and adult animals may gain or lose weight considerably without proportionate skeletal changes (*Gordon et al. (1947)*).

By defining chondrotrophin as a hypophyseal agent that produces skeletal growth, we expect clarity of conception in this field, where other difficulties are enough even without reference to presumably different things by vaguely synonymous terms.

One of the difficulties to which we allude is the lack of a standard for reference (*Marx et al. 1942*). Limited supply of hypophyses and inconsistent clinical results explain a lack of initiative to establish a standard. Standard preparations promote, however, mutual understanding, somewhat like dictionaries bridge some of the gaps between different languages. Standards cannot be replaced by relying on certain points in the procedure of assay, by statistical evaluation of results, not even by crystalline chondrotrophin (*Li et al. (1945)*, *Li (1947)*, *Fishman et al. (1947)*). It is difficult to reproduce extreme refining and even then, physical and chemical constants do not warrant identical activity in proteins. In the steroid class of preparations whose constants are more informative, the concentrated effort of more than a decade was needed for a fair degree of security in the identification of products showing definite types of activity.

PRINCIPLE OF THE TAIL TEST

Chondrotrophin is assayed in our laboratory by comparison of pre- and post-treatment caudal skiagrams of hypophysec-

tomized rats. The tail length is equal to the sum of 27 vertebrae, intervertebral spaces and skin tip. In rats aged 50 days the majority of 54 epiphyseal disks (in the distal vertebrae of the tail) is unossified. Comparison between tail and other skeletal parts was convincing to show that the tail length truly indicates the general skeletal response. The advantage of the tail is its straight shape and its far greater growth than of any single bone of the rat. (See plate, pag. 96).

Agreement is not unanimous as to the tail test deserving priority above other assay criteria. Opinion is however growing unanimous as to the chondral point of attack of chondrotrophin (*Evans et al.* (1943), *Ingalls* (1941), *Ingalls et al.* (1941), *Becks et al.* (1946), *Griffiths et al.* (1942)) though the importance of other endocrines for the chondral reaction has been emphasized by the groups of authors just quoted and by ourselves (*Evans et al.* (1939), *Laqueur et al.* (1941)).

Chondrotrophin is assayed by *Evans et al.* (1943) by the short (4 days) test, consisting in measurements of the proximal tibial epiphysis in rats whose hypophysis was removed more than 12 days prior to their substitution cure.

CLINICAL ATTEMPTS

Few firms ventured into the production of »growth hormone« and we tested almost all the preparations on the market or supplied on request as they appeared. With one recent exception, we never succeeded to detect chondrotrophin activity in these products. Instead of detailing these results here, suffice it to state that those tests were interspersed between tests of our preparations of chondrotrophin against which the foreign preparations stood out as consistently inert.

When reviewing these findings, we are unsurprised by discrediting clinical reports on »growth hormone« (*Shelton* (1942)).

It is fair to state, however, that our own preparation whose activity was meticulously tested and a posteriori controlled, also failed to yield the promise of reliable action. Our clinical

attempts comprised a group of 27 patients under the care of expert clinicians.

Between 1936 and 1941 we accumulated these clinical data. A part of the patients has been referred to our laboratory by the Medical Service of the N. V. Organon, whenever an inquiry was received there as to the availability of a potent preparation. Among all these medical contacts, we distributed nearly 7000 ampoules of chondrotrophin. Most of the ampoules were charged with 100 ($\pm 20\%$) »units« (see below) and a few with 1000 ($\pm 20\%$) »units«. Merthiolate (Lilly) was added as a preservative in 0.1 per thousand concentration. The concentrated solution proved to be unstable when retested for final control. More than 8/10th of the potency was lost. Therefore it is valueless to distinguish two concentrates. This experience shows the difficulty of maintaining highly concentrated chondrotrophin in solution.

The 27 cases studied represented a selection from 94 cases about which there was correspondence. They were cases of nanism in both sexes. Many of them were declined as unsuitable for our work owing to various reasons. Among these reasons age was prominent. Patients above 21 years, with closed epiphyses were considered to have no chance to grow. Many cases were above 15 years of age. We supplied chondrotrophin to 58 cases. It was surprising to experience the reluctance of doctors in various countries to supply adequate information on their cases. In more than half of the cases to which we supplied material (free of charge), not even the sex of the patient became known to us and of many cases it remained unknown if they ever were injected. There is perhaps a difference in attitude when practitioners are supposed to cooperate in an experiment, or when they are merely asked to collect the harvest of application of a product whose mode of action is more dramatic and better predictable than that of chondrotrophin.

One female patient is not included among the 58, not because she was declined from supply, but because she was dwarfed by starvation and restored to satisfactory growth by

diet alone (Dr. J. Groen). We mention this case merely to show the variations in aetiology of dwarfism.

In order to evaluate our results, we made up a table from data of *Engelbach* (1932) available at the time. Moderately growing persons begin at 0 age at a length of 51 cm. to grow by yearly increments as shown in table 1.

Table 1.

Increments of length in humans in cm. per year between 1 and 18 years of age. From 10 to 18 years 1 cm. may be added per year to the figures for males.

Year.....	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Increment.	23	11	9	7	6	6	6	6	6	5	5	5	5	4	4	4	2	2
Length....	74	85	94	101	107	113	119	125	131	136	141	146	151	155	159	163	165	167

In all but 8 cases, the observation was confined to 3 months. We decided to evaluate all the results on a quarterly basis.

We advised two series of 30 subcutaneous injections (one ampoule each) with one month interval. We emphasized the need of a liberal diet with Ca, P and vitamin D supplement in addition to other minerals and vitamins. Body length was measured in the morning before and after the observation period.

From the table we noted:

The *age* corresponding to the given length.

The *length* expectation at the given age.

The difference between length expectation and real length (growth delay), was divided with four times the difference between true age and »length age«. The quotient gave the quarterly growth delay. Patients growing during the quarter of observation more than their quarterly growth delay were listed in the group of presumable responses. For example: a boy of 10 years was long 117 cm. He should have been (according to our table) either 7 years old ($10 - 7 = 3 \times 4 = 12$ quarter years behind time), or long 136 cm. ($-117 = 19$ cm.

growth delay); his quarterly growth delay was therefore $19/12 = 1,5$ cm. This patient grew 3 cms. during the quarter of observation, he was a presumable response, the more because 3 cm quarterly growth would occur at an age of 2 years and not later (see table 1).

Six of such presumable positive responses were secured out of 27 cases. We are reluctant to assign more than a very cautious credit to chondrotrophin.

There were no untoward reactions.

In addition to total length, we also examined tibia skiagrams and attention was directed towards dental development. Chondrotrophin may as well favourably influence both dentitions (a point specially emphasized by our associate of the time, *L. H. Levie*).

The general impression of our series is disappointing and there may be several reasons to explain that.

A) Many aetiologies may underly dwarfism, chondrotrophin may be adequate to deal only with a few of them.

B) Our largest doses represented one or a few bovine adenohypophyses per injection which may be inadequate for human growth stimulation.

C) Bovine hypophyses may be unsuitable for human growth stimulation, as they are unsuitable for gonad stimulation in rats. In hypophysis research species matching is more than a ready escape from disappointing experience.

D) Our preparations may have been concentrates of a poorly active chondrotrophin metabolite, occurring in excess in hypophyses, whereas true chondrotrophin might be as much more potent as it is scanty. Moreover the most potent chondrotrophin may have escaped concentration in our extracts (see below).

We remind of our own experience in the steroid class of agents. Testosterone (and the augmenting X-substance) was discovered in our laboratory after accumulation of evidence to show that urinary androgens were mere steroid metabolites of low potency and of doubtful origine. The potency problem (a species problem as our example has shown) may be compli-

cated by a limiting factor not itself active, but ruling over the potency of the active agent.

E) These considerations lead to the last point that we wish to mention here, namely synergism between chondro-trophin and other hypophyseal agents, more necessary perhaps in human dwarfs than in hypophyseoprivic rats.

STANDARD, ASSAY

In a short paper we described our standard and chondro-trophin assay (*Freud & Dingemans* (1946)). Since 1939 we used the same acetone dried powder of freshly frozen bovine hypophyses, Gr 238 R) as a reference preparation. Of this powder 0.35 mg were defined as a *unit*. The unit, or a multiple of it was dissolved for daily injections in 0.05 to 0.2 ml. water at p_H 10.5 and the centrifuged solution decanted from its insoluble residue. Close to 2/5th of the powder was soluble.

Rats were used when hypophysectomized on the day of, or a day before the first injection. Though irrelevant for the action, we mostly injected intraperitoneally. Cold stored extracts may otherwise cool too small an injection site and produce skin necrosis and sloughing. Our groups consisted of 5 rats, littermates being distributed over parallel groups. In the rat colony and in the experiments the rats were fed the same constant diet, though the mixture had to be altered twice owing to the war. All three mixtures used were fairly satisfactory as to fertility and growth maintenance. The worst was the »war-mixture«, owing to its low essential protein content.

Daily injections were continued for one week or a fortnight.

The unit aequivalent of the unknown preparations was calculated on the basis of their nearest to aequipotent doses with one of the doses of the standard. Exclusively simultaneous experiments were evaluated. Aequipotency was assumed only between two responses above a predetermined threshold. The threshold requirement was more than 6 mm. tail growth in the majority of group mates in the experiments of one

week duration. A 1.5-times higher minimum requirement was applied to experiments of two weeks.

Body weight and tail length often failed to show parallel responses and they varied, again without detectable parallelism, with the season. These variations gave sometimes the impression that they would correlate with the length of the daylight.

They were unpredictable, however, as statistical attempts have shown with yearly groups of up to 325 rats whose treatment was done with the same dose of the standard. It is a different thing to adjust a calculated dose to a definite seasonal sensitivity, or to roughly adjust doses to the seasonal response expectation. Lately we adopted the policy of giving 2 and 6 units of the standard per day, either to simultaneous groups in the transitional season, and 2 units in the responsive (long day) and 6 units in the non responsive (short day) season. Of the unknown the equipotent doses are considered also to be 2 or 6 units. We omit dose/action ratio calculations, since we realise that truly computable material is seldom in experiments performed in a limited number of rats, consistent with a reasonable performance at the hypophysectomy table. The threshold type of assay has certainly great advantages, including accuracy, for this type of work.

We attach importance to three classes of data.

- I. Doses expressed as acetone dry powder equivalent (yield).
- II. Actual dry weight given per daily injection (purity).
- III. Potency expressed in terms both of I and of II.

In seven years (up to 1946) experience was collected with the standard (Gr 238 R) by testing it in 1257 hypophysectomized male rats. The same standard is still in use.

There were 184 untreated controls.

622 rats injected as specified in table 2.

635 rats were excluded from the statistics for one of the following reasons.

Rats belonging to groups smaller than 30 membered when used for doses intermediary to those mentioned in the table.

Incomplete hypophysectomies in near to 10 % of the cases, as ascertained at autopsy or in doubtful individuals histologically (in sagittal sella sections).

Rats dead before the conclusion of the experiments (15 %).

Table 2 shows the results in the controls and in the 622 rats used for the statistics, in total 806 rats.

Table 2.

Referring to 806 hypophysectomized male rats, whose average weight at operation was 100 Gm and whose tail length at that date varied between 120 and 150 mms.

Tail length increments in mm. Treatment with chondrotrophin and untreated controls.

Prepar. Gr 238 R mg dose/ day	After one week			Significance	After two weeks		
	Nr. of rats	Av. tail incr.	Mean error		Nr. of rats	Av. tail incr.	Mean error
0	75	3	0,22	0,66 $3 \times 0,28$	109	3,66	0,17
	↕	1,8	$3 \times 0,37$		2,74	$3 \times 0,55$	↕
0,175	61	4,8	0,3	1,6 $3 \times 0,60$	43	6,4	0,52
	↕	2,43	$3 \times 0,37$		2,47	$3 \times 0,37$	↕
0,35	135	7,23	0,21	1,64 $3 \times 0,42$	95	8,87	0,37
	↕	0,67	$3 \times 0,44$		4,43	$3 \times 0,46$	↕
0,7	95	7,9	0,39	5,4 $3 \times 0,48$	110	13,3	0,29
	↕	2,6	$3 \times 0,69$		2,81	$3 \times 0,64$	↕
1,4	20	10,5	0,58	5,61 $3 \times 0,81$	53	16,11	0,57

In this table 2 the double pointed arrows mark the characteristics of significance. A difference between two average tail increments is considered significant if it is more than three times the combined mean error of these averages. The combined mean error is, as usual calculated by the formula

$$\sqrt{Me_1^2 + Me_2^2} = \text{combined Mean error (Me)}$$

Where differences are significant, fat print has been used.
Our main conclusions were:

1. Acetone dried bovine hypophyses yielded potent and constant chondrotrophin for years.

2. Hypophysectomy arrested tail growth within a week in male rats as specified here. Their spontaneous tail growth remained between the limits of 3 to 4 mm, which is well below our threshold requirement.

In an earlier paper (*van Eck & Freud (1941)*) we have already contributed data to the problem of growth without hypophysis, when we have shown, that the earliest growth of the skeleton and the earliest gain of weight seem ahormonal in rats. The hypophysis gains control gradually. It was comparatively irrelevant for growth at the age of 9 days (when the experiment began) but in 5 weeks time there was a tail growth delay of 65 mms with reference to rats of the same size and of 118 mms. with reference to rats of the same age, yet the tail had doubled its length with reference to the 8 mms it had been on the day of operation. This result was again a thorough confirmation of the value of the tail for estimating chondrotrophin. The ahormonal tail increment was lagging well behind the weight increment.

3. In large groups 0.175 mg of the standard produced significant growth, yet not reliable enough in groups of 5 rats. That was our reason of choosing 0.35 mg as our unit of the standard.

4. One and two units were indistinguishable in one week, but their action was significantly different in two weeks. The ratio between dose and effect was also more evident in two weeks than in one week. Therefore (though we do not use dose/action diagrams for assay estimates) we prefer fortnightly experiments.

5. The dose effect ratio being as antilogarithm to logarithm, it seems preferable to let the dose rise by triplication in order to obtain significantly different responses. Thereby the inac-

curacy of the assay is admitted in advance up to the limits of the chosen dose differences. Using small groups of rats, as feasible under ordinary laboratory conditions, there is no possibility to obtain greater accuracy certainly not by more elaborate calculations.

6. High protein diet, as introduced after the war, might have had a favourable influence upon the responsiveness of rats to chondrotrophin.

ASSAY EXAMPLES

With the assay method as outlined in the preceding paragraph, we tested both new batches of hypophyses and fractions as they arose when refining was attempted. The essential aspects of those attempts were previously published and surveyed in a review of *Freud, Laqueur & Mühlbock* (1939). The final dry residue was in some of the most successful experiments 16 to 35 times and even up to 60 times less per aequivalent amount than in the crude state. Occasionally, when powders with high yield were available, the unit was well below 0.01 mg. This was achieved when 0.35 mg crude acetone powder contained the unit. It is unfortunate that absorption and elution is such a delicate procedure that it would not constantly yield the same result and that it seems unsuitable as a routine refining procedure.

As an illustration of chondrotrophin assay we insert here the abbreviated results with two preparations in table 3. Every line in this table represents 5 hypophysectomized male rats of 90 to 110 Gm initial weight (average 100 Gm) and 120 to 150 mms initial tail length. The injections were given twice a day intraperitoneally during 14 days. The first column gives the experiment number, the second: preparation number (batch), the third: the daily dose in terms of acetone dried powder equivalent, the fourth: the actual weight of the daily dose (two injections) dissolved in 2×0.1 ml. weakly alkaline water, the fifth: the type of processing, the sixth: the weight increment (uncorrected average), the seventh: the tail length increment (uncorrected average), the eighth: remarks.

Table 3.

Weight increments in gm. and tail length increments in mm. of hypophysectomized male rats treated with extracts of hypophyses and fractions thereof as they arose by refining.

Exp. Nr. (month)	Prep. Nr.	mg/day Ac. powd. equiv.	mg/day dry resid. in 0,2 ml.	type of processing	Aver. wt inc- rem. in Gm.	Aver. tail length increm. in mm.	Remarks
63319 (Sept.)	El	0,35	0,140	pH 10,5 extract	none	7	
"	El	0,70	0,280	" " "	10	11	
"	Gr 238 R	0,35	0,140	" " "	8	11	
63320 (Sept.)	El	0,35	0,015	Ads/Elut.	-2	2	1 dead
"	El	0,70	0,030	" " "	1	6	2 dead
"	Gr 238 R	0,35	0,140	pH 10,5 extract	-1	8	
"	Gr 238 R	0,70	0,280	" " "	3	7	1 dead
63322 (Oct.)	El	0,70	0,280	pH 10,5 extract	9	13	
"	El	2,1	0,840	" " "	12	13	
"	El	0,7	0,030	Ads/Elut.	3	7	
"	El	2,1	0,090	" " "	none	7	1 dead
"	0	0	0	0	-3	5	untr. contr.
63323 (Oct.)	El	3,5	1,4	pH 10,5 extract	15	17	2 dead
"	0	0	0	0	-6	4	untr. contr.
"	0	0	0	0	39	29	unop. controls
63325 (Nov.)	El	0,70	0,280	pH 10,5 extract	11	13	1 dead
"	El	2,10	0,840	" " "	16	21	
"	El	0,70	0,050	Ads/Elut.	0	6	1 dead
"	El	2,1	0,150	" " "	1	9	2 dead
63326 (Dec.)	El	0,70	?	Ads. resid.	18	9	1 dead
"	El	2,1	?	" " "	13	11	
"	El	0,7	0,280	pH 10,5 extract	19	14	1 dead
"	El	2,1	0,840	" " "	24	17	
83342 (Febr.)	W. C. 1.	2,1	0,132	pH 6,8	15	12	
"	W. D. 1.	2,1	0,065	24% aethanol	10	10	1 dead
"	0	0	0	pH 4,6			
"	Gr 238 R	2,1	0,840	24% aethanol	-6	3	Sham treatment
				pH 10,5 extract	13	14	1 ill

Table 3 refers to experiments in 135 hypophysectomized rats. 14 of these rats died before the end of the experiment and one was deduced from its group owing to apparent illness. These experiments were performed in the years 1946 to 1948. The results show that EI was an acetone powder of acceptable potency, not essentially different from our standard. This will be more eloquently demonstrated in the last paragraph.

Every experiment number heads groups of simultaneous experiments, and only such experiments afford direct comparisons.

After having shown that EI had a useful potency, we proceeded to use a crude extract of this preparation as its own reference for comparison with attempted refinements. The eluates in experiments 63320, 63322 and 63325 were a poor success, because the loss of potency was great and because the unadsorbed residue (exp. 63326) contained at least as much activity as that adsorbed and subsequently eluted. We are inclined to ascribe this failure to differences in the quality of the acetone dried powder as compared with earlier experiments with other powders when they were subjected to the same procedure with the same adsorbents, even the same batch of coal.

The experiments quoted under number 83342 were performed with fresh bovine hypophyses. They were treated as described in the paper of *Fishman et al.* (1947). Both yield and purity were more satisfactory than the adsorption experiments in the first part of the table.

While the standard of supplementary crude reference preparations afford an opportunity to estimate the potency of derived products, the untreated controls (in exp. 63322, 63323), sham treated controls (exp. 83342) on the one and the unoperated controls on the other hand (63323) are useful to give an impression of the measure of substitution obtained with the experimental fractions. They show, that these assay experiments remain far below complete substitution.

In actual practice we often pay attention to littermates in parallel groups and to individual variations. The pertinent

data could not be included in the table without undue demands on space.

TAIL AND TIBIA EPIPHYSIS IN CHONDROTROPHIN ASSAY

Chondrotrophin keeps epiphyseal junctions open through eutrophy of the cartilage. Such eutrophy is a prerequisite of the bone shaft development by progressive ossification. In long bones the cartilage plate separates the bone head (epiphysis) from the shaft. There is polarity in the epiphyseal junction, because even in normal longitudinal growth the bone head side of the plate much sooner ossifies, than the diaphyseal side.

As there is aequilibrium between cartilage eutrophy and ossification it is evident, that chondrotrophin can achieve only a limited thickening of the cartilage plate. This was confirmed by the facts collected in experiments in which we measured the proximal tibial epiphysis in treatments of two to three weeks with widely varying doses and computed the results with the tail measurements. For these experiments nearly 100 rats were used, some of them untreated, others injected with rising doses. Tail length increment and epiphyseal width were roughly parallel in the range of moderate responses. In control (untreated or ineffectually treated) rats 6 to 8 ocular micrometer units were measured as tibial epiphyseal width. The maximal measurements were 14 to 16 comparable micrometer units. The tail length was in the same rats increased by a maximum of 5 mms. without effective treatment and by 12 to 40 mms at various effective dose levels.

As the authors (*Evans et al. (1943)*) of this method rightly state, its main advantage is shortness and the use of little chondrotrophin. No consistent results would be obtainable with the tail test in such a short time. Tail growth increases with the dose and the length of the treatment, whereas the epiphyseal cartilage depends mainly on the daily dose and once at its top width, it can respond with no more increment. In many of our rats we might have discontinued treatment

10 to 17 days earlier yet no lower micrometer figures would have been obtained. It is subject to no doubt, that chondrotrophin is being estimated by this test.

THE PROBLEM OF ACQUIRED REFRACTORINESS

Rats are known to develop refractoriness to gonado- and to thyrotrophin. Bovine hypophyses are a poor source of gonadotrophin for rats and there is no conclusive evidence available that this type of gonadotrophin would elicit transferable refractoriness in them.

Intraperitoneal injections of bovine hypophyseal extracts are acutely antigonadotrophic against simultaneous subcutaneous treatment with every known gonadotrophin, but this is not a serially transferable refractoriness. Some other mechanism underlies this puzzling phenomenon.

We performed a few experiments with bovine chondrotrophin in rats with a view to test it as to its antigen action.

Ten female rats of 30 to 40 Gm weight at the age of 28 days, were injected 148 times in 200 days with 25 mg equivalent of Gr 238 R (the standard) each time. This was a dose corresponding to 71 units per injection. The experiment lasted from 17 Sept. 1945 till 7 March 1946. The final body weight of one of the rats was 216 Gm, the other nine were between 256 and 306 Gm. The tail length was 83 to 100 mms at the beginning and 187 to 216 mms at the end. The growth of these rats corresponded in every phase with the usual average as did their tail growth. Their weight increment was unlike plateaued rats when their weight reached 250 Gm between 3 and 4 weeks prior to the conclusion of the treatment. Their final hypophyseal weight was low (10 mg average), the thymus was large for their age (246 mg average). The ovaries and sex accessories were variable of size, not involuted, thyroids and adrenals were of normal size (23 mg and 80 mg average). These rats were neither giants nor did they supply indications for refractoriness either to chondro- or to gonadotrophin.

They were bled by decapitation and 0.3 ml. of their serum was subcutaneously injected daily to tail test rats along with

one unit intraperitoneally of the standard (0.35 mg of Gr 238 R) during 7 days. Controls were treated with the standard alone. The average gain of weight in 5 serum treated rats was 5 Gm in a week, with a tail increment of 9 mms, while in the five control rats the corresponding figures were 3 Gm and 10 mms. There was therefore no significant transferred refractoriness.

We then turned to another type of experiment on the same subject. Daily injections of 25 mg acetone dried powder equivalent of the standard were given to 20 male rats for three weeks (14 Jan. 1946 to 5 Febr. 1946). Then these twenty rats and 5 comparable controls were hypophysectomized and injections were now given daily to all rats, but the dose was reduced from 71 to 5 units (1.75 mg acetone dried powder equivalent of the standard) per day. All the injections were given intraperitoneally. The average weight increment of the 20 pretreated rats was 15 Gm with a tail growth of 11 mm in a fortnight, while the five controls gained an average of 20 Gm weight and 12 mm tail length. Since these differences are non significant, these experiments should be considered either inconclusive or contrary to the assumption of acquired refractoriness to bovine growth hormone in rats.

It should be understood, that these experiments contain no indication as to other than bovine chondrotrophin in rats.

It is noteworthy, that we regularly notice a decrease of tail length increment in the second and especially in the third week of treatment of hypophysectomized rats. It was always a problem, whether an equilibrium is approached between a given daily dose and the tail growth already achieved, or if refractoriness would develop as a result of treatment during one week. This question seems to be settled now, so far as the present type of chondrotrophin is concerned. Rats comparatively heavily pretreated, again responded a little better to the first weeks' injections than to those of the second week, just like the non pretreated controls. The decrease in response may be due to deterioration progressing after hypophysectomy while substitution is inadequate.

RESTITUTION

Our attempts to produce gigantism in rats met with moderate success. Of several experiments we reported one of 8 months with daily doses of 70 units, whose dry residue was 4.2 mg aequivalent to 35 mg acetone dried powder (*Freud, Dingemanse & Levie* (1939)). Among the skeletal responses in that experiment, pelvic enlargement was rather striking.

Subsequently *Freud & Dingemanse* (1940) when reporting on hypophysectomized rats stated that 235 units of chondro-trophin daily produced more than normal growth, namely weight increments of 80 to 96 Gm (in 4 out of six rats) and tail length increment above 40 mms in the same rats. The dry residue per daily dose was in the two preparations used for that experiment 8.23 and 6.4 mg. The unit was therefore contained in 0.035 and in 0.028 mg. The acetone dried powder contained the unit in not too far from 0.35 mg and its weight was reduced by salting out and by pH manipulations to less than 1/10th.

These two experiments show, that more than three times as many units per day were needed for restitution of growth after hypophysectomy, than we tried to use in the attempt to produce excessive growth. We might have anticipated the failure to achieve gigantism. It may well be that for stimulation of excessive growth in normal rats more than the restitution dose is needed. This conclusion is in reasonable agreement with experiments published from the laboratory of *H. M. Evans* by *Li & Evans* (1947).

These authors report on a pure growth hormone, whose unit (not far from ours) is contained in 0.01 mg. They injected up to 200 times that much in a long experiment to obtain excessive growth. The actually injected dry residue was accordingly 2 mg per day. From 1 Kg. fresh bovine adenohypophyses they obtained 50 mg pure substance, that is 25 daily doses. Their yield of acetone dried powder from mashed hypophyses was 250 Gm. Their refinement amounted to a reduction of dry residue 5000 times. Their daily dose was according to

this calculation the equivalent of 10 Gm acetone dried powder (in the latter part of the experiment).

While the agreement in daily units between our restitution and the Californian dose for gigantism is rather remarkable, it is obvious from the figures here presented, that for about 82 mg acetone dried powder of our laboratory the Berkeley workers used 10,000 mg of their acetone powder, which is more than 100 times as much.

We had an opportunity to discuss with Professor *Evans* the problem of growth hormone early in 1947. His statement in answer to our question of losses in the process of purification was, that losses were negligible, though difficult to assess, as crude preparations are less potent on account of contaminating active principles interfering with growth. It seems improbable, that this antagonism would reduce the growth principle to 1/100 of its activity. We have no explanation for the remarkable fact, that bovine adenohypophyses at Berkeley yield 5000 units per Kg and that they yield 500,000 or even more similar if not quite identical units at our laboratory. We are more than ever convinced, that data on yield are as essential as are data on unit dry weight.

We were not in a position to refine a sufficient amount of chondrotrophin for the purpose of reproducing at last experimental gigantism in rats. But we had enough of the preparation El (see table 3), thanks to the courtesy of Organon Ltd., London, to perform a series of complete restitution experiments with the crude extract of that acetone powder. These experiments lasted 3 weeks. It was assumed, that 0.35 mg of the powder would be a unit of chondrotrophin, though according to Table 3 this may be a little too optimistic. We injected twice 0.5 ml per day, 1 ml containing 200, 400 or 800 units (70, 140 or 280 mg equivalent of the acetone dried powder) to recently hypophysectomized rats of the same type as described in preceding paragraphs. There were two kinds of controls, namely hypophysectomized uninjected and sham operated untreated rats. The results are averaged and thus

computed in table 4. Dead rats are omitted from the table. The experiments were performed in February, March and December 1947.

We used 50 hypophysectomized rats for treatment, 20 for control and 20 non hypophysectomized rats.

Table 4.

Weight increments in Gm and tail length increments in mm. of hypophysectomized male rats treated with chondrotrophin for 3 weeks, as well as untreated controls and untreated sham operated controls.

Daily dose in units	Number of rats	Aver. init. weight Gm	Weight incrim. in Gm.	Tail length increment aver. in mm	Simultan. experim. bear ident. roman figures
Hypophy- sectomized					
200	8	111	27	18	I
200	7	101	42	28	II
400	7	98	61	34	II
400	5	113	65	29	III
800	6	106	45	27	IV
Hypophy- sectomized					
0	4	118	—4	2,6	I
0	4	105	—4	6	II
0	5	129	—2	4	III
0	4	107	—9	3	IV
Sham operated					
0	5	113	59	29	I March
0	5	107	90	42	II December
0	5	110	62	31	III February
0	5	111	60	29	IV June

This table shows, that normal weight increments may vary by 30 Gm in three weeks and that in treated hypophysectomized rats there is a tendency to greater weight increment parallel with normal controls.

The hypophysectomized rats were adversely affected by the injection of 280 mg aequivalent of acetone dried powder

(800 units), they had peritonitis. The dry residue injected was nearly 100 mg a day. A solution of 100 mg per ml was lyophilized till the required high concentration was attained.

In table 4 a. we present the averages of a few organ weights.

Table 4 a.

Weight in mg of organs from hypophysectomized male rats treated with chondrotrophin for 3 weeks, as well as untreated controls and untreated sham operated controls.

Daily dose in units	Groups	Mg weight of Thyroid	Mg weight of				
			Supra- renal	Testicle	Semin. Ves.	Thymus	Preput. gl.
Hypophy- sectomized							
200	I	8	20	563	63	—	79
200	II	12	26	444	71	384	94
400	II	15	35	447	40	430	133
400	III	12	40	834	110	—	154
800	IV	13	35	698	126	202	154
Hypophy- sectomized							
0	I	8	14	186	12	—	20
0	II	6	14	172	9	225	18
0	III	7	14	203	12	—	18
0	IV	8	10	204	12	138	17
Sham operated							
0	I	14	32	1898	287	—	79
0	II	29	42	1916	259	487	83
0	III	11	36	1640	194	—	75
0	IV	18	34	1216	462	240	91

This sub-table shows, that besides chondrotrophin other trophic agents were represented in the extracts, to whose action we ascribe the maintenance of thyroid, suprarenal, testicle and thymus weights. Comparison between the two tables shows, that the best increments were obtained in rats whose thyroid was large, both in treated and controls. The gonadotrophic potency seems to be irrelevant to growth. The suprarenals, thymus and preputial glands are large in rats

which have grown well. The preputial glands are supernormal in the groups injected with high doses.

The general conclusion from these results is, that complete restitution after hypophysectomy (or perhaps more cautiously: maintenance) is evidently feasible with hypophyseal extracts. Despite that, life expectance is less favourable after hypophysectomy than after sham operation (Infection? Adaptation?).

A comparison with tables 2 and 3 shows, that while growth with 4 to 6 units a day is significant in two weeks, nearly 100 times as much is needed per day to come somewhere near complete restitution.

It is seductive to speculate on the causes of the enormous requirement for complete restitution as compared with amounts producing highly significant responses. We have already mentioned the idea, that perhaps a more potent chondrotrophin exists, than that which is active in our assay type of experiment.

Another possibility would be, that other trophic principles, especially the thyrotrophin, may be of major importance for strong growth, while it might be unimportant for such growth as we produce in assay experiments. This point is easily accessible to investigation.

Finally it might be, that chondrotrophin itself acts at two different points of attack. One of these — cartilage — would have a low »differential threshold«. The other point of attack might be in the bone or other tissues relevant for growth. This point of attack might have a high »differential threshold«, perhaps 100 times as high as cartilage. Moderate growth might result from unstimulated cooperation of the second point of attack, whereas strong growth might require a stimulated cooperation and therefore excessive amounts of chondrotrophin or for that part of another trophin existing in the preparation.

On the elucidation of this point may depend a part of the applicability of chondrotrophin for the purpose of human therapy.

We acknowledge with gratitude generous subsidies and cooperation to facilitate our work during the past years by N. V. Organon, Oss, Holland.

SUMMARY

Skeletal indicators, especially the epiphyseal cartilage of hypophysectomized rats, are recommended as decisive indicators of the growth principle, to which the name chondrotrophin rather than somatotrophin is assigned. Weight increment is less characteristic. A standard for reference is wanted. Chondrotrophin comparative assay was based on pre- and post-treatment skiagrams of the tail of hypophyseoprivic rats, which is representative for skeletal growth, since the tail consists of 27 vertebrae with 54 epiphyseal disks.

Out of 27 patients 6 responded with a length increment in excess of expectation during 90 day cures with 100 »units« chondrotrophin per day in two courses of 30 injections. Cautious credit is given to chondrotrophin. The principles of assessing results and causes of failure are discussed.

A local standard acetone dried powder of bovine adeno-hypophyses was used for years. Of this powder 0.35 mg was defined as unit.

Comparative assay results were evaluated on the basis of equipotent yield and purity. Statistics of 806 rats were basic for assay principle: 1, 2 and 6 units of the standard were used for comparison in 7 or 14 days experiments. The threshold tail growth requirement was 6 mm in male rats of 100 Gm at hypophysectomy in 7 and slightly more in 14 days experiments.

Very young rats (9 days) grow ahormonally for days, the growth delay becoming more marked with age progress. Diet and environment have influence. Assay examples are discussed showing detection of chondrotrophin in main and other fractions. Occasionally 0.01 mg purity per unit (60-fold reduction of dry weight) was achieved.

Proximal tibia epiphysis height measurements are valuable short term indicators of activity, but inferior to tail test.

No acquired refractoriness to chondrotrophin (bovine) in rats was demonstrable in two sets of tests (seral transference and pretreatment experiments).

Gigantism was not really obtained perhaps on account of low dosage (70 units) but complete substitution (maintenance of growth) for 3 weeks was repeatedly obtained with daily doses well above 200 units.

These experiments in addition to assay, conclusively show a high yield in crude extracts as compared with results quoted by the Berkeley group of workers.

In our crude extracts there were other trophic principles, especially thyrotrophic in addition to chondrotrophin.

Since the assay dose (semirestitution) is a hundred times below the restitution dose, the possibility of more than one chondrotrophin or more than one point of attack with widely different »differential thresholds« is mentioned for hypothetical explanation.

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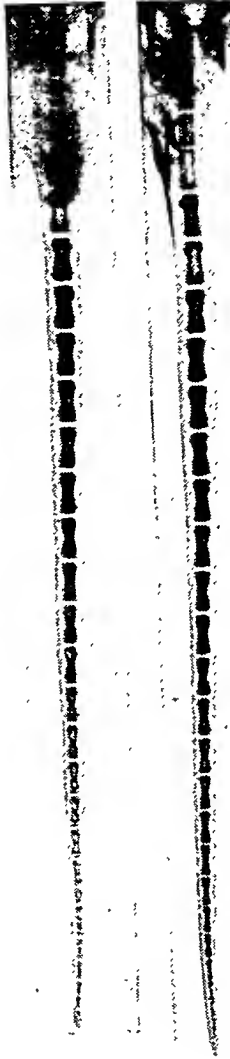
Tail skiagrams of rats.

Interval between skiagrams of the same rat: 3 weeks.

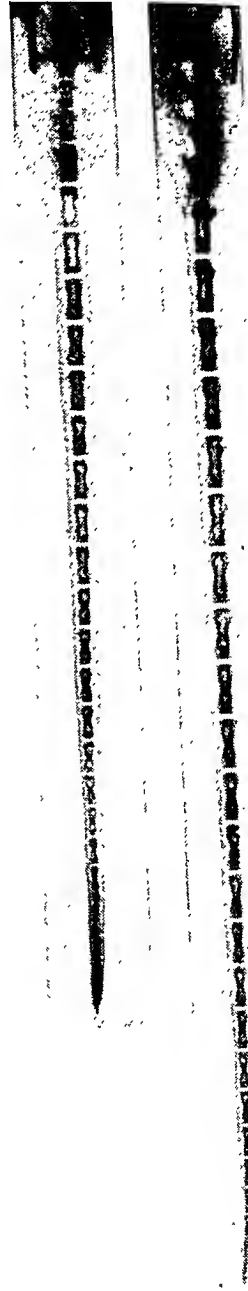
Non hypophysectomized control



Hypophysectomized control



Hypophysectomized rat, injected with 400 units of chondrotophin



From the Pharmacological Laboratory of
the State University, Leiden.

THE GONADOTROPHIC POTENCY OF THE HYPOPHYSIS IN OESTROGEN-TREATED RATS

BY

J. H. GAARENSTROOM AND S. E. DE JONGH

Some years ago *Gaarenstroom & de Jongh* (1943, 1946) supplied *biological* evidence for the joint presence of two gonadotrophic hormones in the hypophysis of the rat. To make the following exposition more easily understandable we will briefly recapitulate the arguments that have led to this conclusion.

It is generally known that in rats the gonadotrophic potency of the hypophysis decreases when the animals are treated for some time with oestrogens. When such animals had received daily 0.2—0.5 mg. oestradiol during a fortnight, the growth of their hypophyses produced of the testes of the hypophysectomized young male recipients in whose abdomen they were grafted, appeared to lag behind the growth that was obtained by means of the hypophysis of an untreated donor. As we knew that the testis requires two substances, viz. the substance described under the name FSH and another one, which is called ICSH, for its growth, we repeated the implantation experiments with recipients to whom in addition a surplus of ICSH chorionic gonadotrophin was given. That this enhanced the effect of the implanted organ is not surprising, but we noticed also something else, namely that there was now no longer any difference between the action of the two kinds of

hypophyses. This might have been due to a supra-maximal value of the gonadotrophic stimulus. When, however, we reduced the size of the implanted pituitary tissue, we found that the effect decreased, and the stimulus therefore could no longer be regarded as supra-maximal. In these cases too the effect of the hypophyses of the oestradiol monobenzoate treated donors nevertheless remained the same as that of the hypophyses of the untreated donors!

When chorionic gonadotrophin is injected in young hypophysectomized rats that have not been provided with another hypophysis, it produces *no* testis growth of any importance. This proves that ICSH can not have been the gonadotrophic substance by which in the experiments mentioned in the preceding paragraph the testis growth was produced. The amount of the substance that was responsible for that effect, underwent apparently no decrease when the animals were treated with oestradiol monobenzoate. Therefore, the substance whose production decreased, must have been ICSH, the other one FSH.

We have thus shown 1° that a high dose of oestradiol monobenzoate decreases in the rat the production of ICSH but not that of FSH and 2° that these two substances are present in the hypophysis independently of each other. This is the position that was reached when we concluded our previous experiments.

It seemed worth while to undertake similar experiments with *female* recipients. A priori there seem to be no reason why the choice of another criterion for the estimation of the gonadotrophic potency of the hypophysis would lead to different results, but a confirmation of our earlier conclusions would, all the same, be wellcome. Moreover, we should not forget that the growth of the ovary does not depend on two but on three gonadotrophic factors, the third one being prolactin, which causes an increase of the cell size of the corpora lutea (*Evans et al.* 1941). It was of interest to investigate whether a treatment with oestradiol monobenzoate would also produce a decrease of the prolactin production in the hypophysis.

In a preliminary experiment the rats in which hypophyses were implanted received no chorionic gonadotrophin after the implantation. The donors were daily injected with 0.2—0.5 mg. oestradiol monobenzoate for a period of 12 to 16 days prior to the implantation. The hypophysis and right ovary of the recipients were removed on the same day, and after that two hypophyses taken either from rats that had been treated with oestradiol monobenzoate or from untreated ones, were grafted into the abdominal cavity. Five days later the left ovary and the uterus were removed. Their weights are recorded in section I of the table, which also gives data relating to a group of rats in which no hypophyses had been implanted.

The table shows that the hypophyses of the untreated donors are the only ones of which a positive effect on the ovary weight is recognizable. The effect on the uterus weight was distinctly stronger than that of the hypophyses of the donors that had been treated with oestradiol monobenzoate.

In the main experiment, in which, because of the additional treatment with chorionic gonadotrophin (2×2.5 i. U. pregnyl of Organon Ltd. daily), a far stronger effect of the implant was to be expected, each recipient received but a single hypophysis. For the rest the experiments were performed in the following way.

Two groups of male rats and one group of female ones were chosen. In each of these groups part of the animals received in the 12 to 16 days preceding the hypophysectomy a daily dose of 0.5 mg. oestradiol monobenzoate. Then the hypophyses were grafted into the abdomen of young female rats whose own hypophysis had been removed. At the same time the right ovary of the recipient was taken away and weighed. Five days later the left ovary was removed and weighed. A histological examination provided data with regard to the presence of corpora lutea (cf. section II of the table, in which the data of these three groups are united).

In all three groups (i. e. independent of the sex of the donors) the effect of the implant on the ovary weight of the receptor is in case the hypophyses were taken from animals

Table 4.
Effects of implantations of hypophyses on ovaries and uterus in hypophysectomized rats.

	number of animals	body weight (g)	nature of implant	additional treatment	ovary weight in mg		classific. of left ovaries (class intervals in mg)				number of ovaries with corp. lut.	uterus weight (mg)
					right	left	0-10	10-20	20-40	> 40		
I	8	51-78	{ oestradiol hypophysis }	—	5½	4½						43
	7	53-80	{ normal hypophysis }	—	6	12						144
	8	50-64	—	—	5	4						21
II	15	67-89	{ oestradiol hypophysis }	pregnyl	8½	28	1	5	8	1	12	
	12	66-89	{ normal hypophysis }	,	8	45	2	1	2	7	9	
III	16	59-82	{ oestradiol hypophysis }	pregnyl	4½	11½	8	6	2	0	3	64
	12	60-83	{ normal hypophysis }	,	4	12½	5	5	2	0	3	93

that had been treated with oestradiol monobenzoate, distinctly lower than the effect on the ovary weight of receptors that had received hypophyses of untreated donors (50—73, 19—37, 16—28; average 28—45 mg.). This result might be ascribed to:

- a. a completely incomprehensible difference between the behaviour of the male recipients with which we dealt in our previous communication, and that of the female ones of the present study, or
- b. the intervention of prolactin.

The choice between these two possibilities is made easier by the outcome of a series of experiments in which recipients were used whose right ovaries were, because of the influence of the season, of a smaller size than those of the rats which we used previously, and whose ovaries reacted therefore less strongly to a gonadotrophic stimulus of the same strength (cf. section III of the table). In *this* group no differences were found in the ovary weights! In contrast to those of the animals used in the other experiments, the left ovaries were, as a rule, *not* luteinized. It seems plausible therefore to correlate the differences observed in the former series of experiments with the presence of corpora lutea.

Now it would be somewhat far-fetched to ascribe a difference in the development of two groups of ovaries that remains confined to the period of luteinisation to a difference in the amount of FSH. The fact that we ourselves are convinced that FSH plays a part in the luteinisation *too*, and have raised for this reason objection against the use of the names FSH and ICSH (= LH) (*Gaarenstroom & de Jongh, 1946*), is of no importance in this respect, for in the period just before the luteinisation we would have a well-marked difference in the development of the ovaries anyhow. That the difference is confined to those cases where the corpora lutea are already present, is, on the contrary, in complete agreement with our second assumption that the difference is due to the action of prolactin. However, against this assumption too some objections can be raised.

In the first place we will have to account for the fact that the histological examination of the corpora lutea of the test animals and of the controls did not reveal a marked difference in the size of the cells. In both groups the corpora lutea appeared to occupy an intermediate position between ordinary corpora lutea and corpora lutea graviditatis. This difficulty, however, need not be disturbing, for although difference in the size of the cells are, as a rule, sufficiently distinct when the begin and end stages of the development induced by prolactin are compared, they are by no means easily distinguishable in the intermediate stages. However, if the difference in the degree of development of the ovary, and here we think, of course, in the first place of a difference in the development of the corpora lutea, should be due to an influence exercised by FSH, we should certainly have to expect a difference in the number of ovaries with and without corpora lutea in the two groups. Section II of the table shows that no difference of this kind is present. If it had been present, it would undoubtedly have revealed itself in these figures.

The fact that the uterus weights in section III of the table show, in contrast to the ovary weights, a distinct difference, seems at first sight a more serious objection against the second assumption. If it is true that the uterus is apt to react more vigorously than the ovary, but it remains all the same rather strange that the uteri of the test rats behaved as if they had been exposed to a lower oestrogen dose than the controls. This would mean that the ovaries of the test rats had produced less oestrogen than the ovaries of the other ones. As no influence of prolactin on the production of oestrogen has ever been observed, the difference in the uterus weights would have to be regarded as an argument against the assumption that the production of FSH has remained the same. The difference in the mean uterus weights in section III of our table, however, can by no means be regarded as significant ($t = 1.4$), as the uterus weights always show a wide range of variability. From a statistical point of view there is, on the other hand, no reason to doubt that the ovary weights in section II of the

table are different and in section III are equal. For this reason we are inclined to accept the prolactin hypothesis for the explanation of our observations.

After suggesting the hypothesis that oestrogen retards the prolactin production of the hypophysis, we have to consider its consequences.

It is generally known that oestrogens may favour the origin of corpora lutea graviditatis in experiments of short duration, and that this effect, which suggests the intervention of prolactin, is not found in hypophysectomized animals. As far as we know, it has not yet been decided whether the effect is due to an increase of the prolactin production or to a stimulation of its activity. The first-named possibility is obviously in contradiction with our hypothesis, but even if this was not so, the second would be a more attractive explanation, because it agrees with our interpretation of the Hohlweg effect.

The Hohlweg effect is a reaction produced in not fully mature rats by a single injection of oestrogen, and consists in the development of ordinary corpora lutea (not corpora lutea graviditatis!). It has always been ascribed to a sudden release of gonadotrophic hormone, but Gaarenstroom & de Jongh (1943) as well as Biddulph & Meyer (1946) could show that it is due to a stimulation of the latter's activity.

At first sight it might seem strange that oestradiol would retard the production of a substance that is so much needed during pregnancy and whose activity it stimulates. This, however, applies, at least to some extent, to ICSH too. During pregnancy the production of this substance is taken over by the placenta, whose activity is in this respect *not* affected by oestradiol. In this connection it is interesting that the placenta can also produce prolactin or, at least, a substance with an identical action (Deanesly & Newton (1941)). A retardation of its production in the hypophysis would therefore not effect the normal progress of pregnancy.

SUMMARY

The effect of implanted hypophyses on the ovary weight of hypophysectomized female rats is decreased when the donors have previously been treated with oestradiol monobenzoate. The administration of an excess of ICSH to the recipients makes no difference in this respect. The decrease may be due to a diminished production either of FSH or of prolactin. The fact that the decrease was not found in male receptors nor in immature female ones in which in the course of the experiment no luteinisation took place, may be cited as an argument against the possibility first-mentioned. When we accept the second hypothesis, viz. the reduction of the prolactin production, the explanation of these facts offers no difficulty, for prolactin is not required for the development of the testis, and it acts in the ovary especially on those corpora lutea that are already present.

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Announcements from the Endocrinological Societies

DANISH SOCIETY FOR ENDOCRINOLOGY

6. Meeting, Jan. 7, 1948, State Serum Institute, Copenhagen.

Aa. Theil Nielsen, K. Pedersen-Bjergaard & M. Tønnesen: Spectrophotometric determination of dehydroandrosterone.

A. Leth Pedersen: A case of adrenal virilism.

7. Meeting, Febr. 26, 1948, State Serum Institute, Copenhagen.

P. Fønss-Bech: A survey of the growth hormone from the hypophysis.

Fridtjof Bang: Some clinical and experimental investigations with regard to the cause of cancer uteri.

C. G. Lund: On the cutaneous resorption of oestrogenic substances from various ointment bases.

8. Meeting, April 29, 1948, State Serum Institute, Copenhagen.

K. Pedersen-Bjergaard, & M. Tønnesen: The urinary excretion of oestrogenic substances and of gonadotrophin in normal women.

M. Bjørneboe, Chr. Hamburger & P. M. Jersild: On the excretion of orally administered oestrone in patients with hepatitis.

SWEDISH SOCIETY FOR ENDOCRINOLOGY

Meeting, Febr. 9, 1948.

U. Borell: The activity of the thyroid gland measured with radioactive phosphorus.

Hj. Holmgren: The innervation of the thyroid gland.

C. G. Bergstrand: The cholesterol in the adrenals, particularly with regard to the effect to testosterone on the adrenal cortex.

C. A. Gemzell: The effect of oestrone on the adrenal cortex.

Meeting, March 15.

L. Thorling: Report of a case of operated insuloma.

B. Hallgren: Report of a case of hypertrophia mammae acuta.

N. B. Nordlander: Patients with insulin resistance.

J. Waldenström: Actual metabolism problems in morbus Cushing.

N. Törnblom: Acromegaly or osteodermatopathie hypertrophiante.

Meeting, April 19.

O. Garm: Studies on cystic ovary degeneration in cows, particularly with regard to its etiology and pathogenesis.

P. Meschaks: Determination of 17-ketosteroid in urine in cows and horses.

S. Nilsson: On dermatosis in the dog, caused by endocrine disturbances.

S. Dyrendahl: Long time investigation with iodinated casein (thyroprotein) on animals in growing ages.

Meeting, May 24.

R. Luft: Carcinoma mammae and testosterone propionate.

H. Fernandez-Morán: The cells of the anterior pituitary in the electron microscope.

G. Klein: The inhibition of the effect of thyroxin on serum lipase by thiouracil.

L. Goldberg: Dextrose tolerance test.

R. Luft: The effect on blood pressure of desoxycorticosterone acetate and salt. I.

B. Sjögren: The effect on blood pressure of desoxycorticosterone acetate and salt. II.

G. Malmström: The effect of testosterone propionate in effort angina.

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PRECOCIOUS SEXUAL DEVELOPMENT PRODUCED BY AN INTERSTITIAL CELL TUMOR OF THE TESTIS

BY

PHILIP SANDBLOM

This is a report of a case of precocious sexual development in a boy 3 years of age, due to an interstitial cell tumor of the testis. Of the eight cases of this type reported in the literature (table 2) this is the youngest and the only case in which the 17-ketosteroids have been studied.

CASE REPORT

In May 1946 a boy aged 2 11/12 years was admitted to the pediatric department of the Crownprincess Louise's Children's Hospital (K. L. V. 528/46). He was the only child of healthy parents. The boy had developed normally until the fall of 1945. Then, at the age of 2 3/12 years he began to increase abnormally in weight and height. In 5 months he gained 4 kg. and in the following 3 months he gained another 4 kg. His facial expression became coarse and his skin became seborrheic. Coincident with this his voice began to change. At the age of 2 8/12 years the external genitals were noted to be abnormally large and he became sexually sensitive with frequent erections. Two months later the pubic hair began to grow. He became difficult, ill-tempered and easily irritated. He was no more interested in playing with other children.

Physical examination (Photo: Fig. 1).

A boy of 2 11/12 years. His height is 108 cm. (the normal 96.7 ± 12.0 cm.) and his weight 21.7 kg. (the normal for the height 18.1 ± 3.9 kg.). The

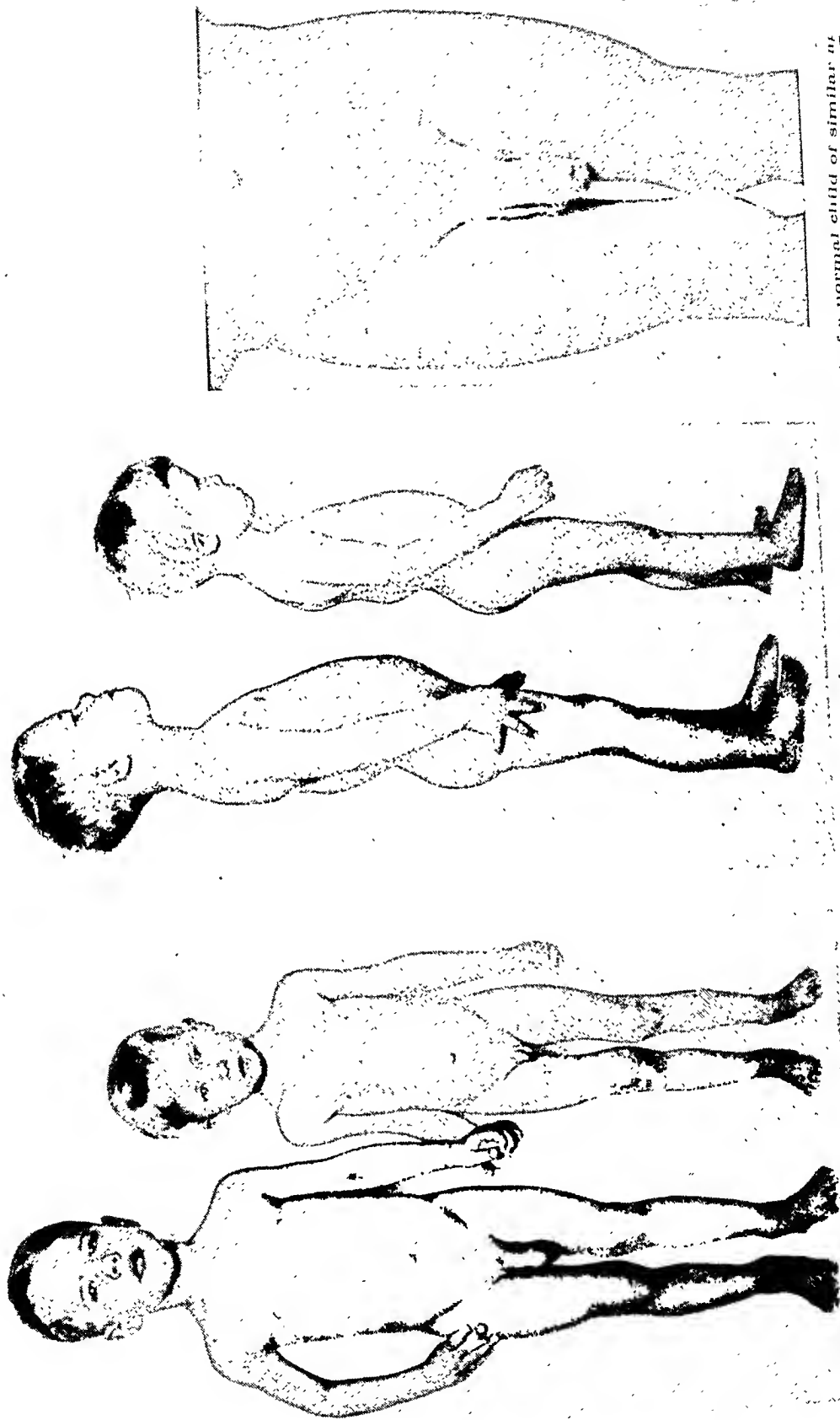


Fig. 1. Patient, aged 2 9/12 years. Note the marked sexual and somatic development, compared to that of a normal child of similar age.

head measures 52 cm. and the chest 60 cm. The lips are thick. No signs of acromegaly. The skin of the face is moist and seborrheic. The voice is as startling as his appearance — a low basso with frequent breaks. The musculature is unduly developed for his age and very firm. X-ray examination reveals the skeletal development characteristic for a boy of from 6 to 7 years of age. Teeth 10/10.

The penis, when flaccid, is 6 cm. The left testicle is the size of a small plum, the right testicle barely half this size. There is some soft hair over the pubis. No hair in the axillae or on the chest. The thyroid is not palpable. The sella turcica is normal. No signs of adrenal tumor are demonstrable either by palpation or by x-ray of the kidneys. Examination of the heart and lungs is negative. The reflexes are normal.

Laboratory findings: Blood studies showed Hb., 75 %; red cells, 4 millions; white cells, 6,200; differential count: stabs 6, segmented 34, eosinophils 3, lymphocytes 50, monocytes 6, and plasma cells 1. W. R. neg.; non-protein nitrogen, 36 mg. per cent; sodium 248 mg. per cent (normal 280—360); potassium 27.6 mg. per cent (normal 17—23); cholesterol 150 mg. per cent. Sedimentation rate 15 mm.

Results of blood sugar tolerance test in mg. per cent: fasting, 120; 1 hour, 156; 2 hours, 132; 3 hours, 104; 4 hours, 76.

The 17-ketosteroid output is 23 mg. per 24 hours. The normal value for this age with the method employed is around 2 mg. per 24 hours.

The intelligence test gave an intelligence quota of 111.

The endocrinologist consulted suspected an adrenal tumor.

It was decided to explore the adrenals; this was done June 5. The right adrenal was entirely normal whereas the left adrenal was firmer and had a dark induration the size of a pea. The left adrenal was removed. The recovery was uneventful.

Pathologist's report. (Prof. H. Bergstrand).

»Three specimens of the adrenal were examined. One of these showed the general architecture of the adrenal. In some areas the medulla is very wide and the cortex thin in proportion. No glomerular zone is seen in the cortex. The reticular and the fascicular zone are of the same width. In the reticular zone some cells have as usual taken some staining with the Ponceau-Fuchsin stain. Outside and within the adrenal capsule some small heterotopic foci of adrenal tissue can be discerned having the character of the glomerular zone.

In the other specimens the architecture is deranged and in a large area of the cortex the capillaries are widened and the parenchyma reduced. In some areas blood has escaped amongst the cells of the reticular zone; in such areas the parenchymal cells show

atrophy or have disappeared and the nuclei are pycnotic in several instances. No blood pigment is observed. Within this area the parenchymal cells take more Ponceau-Fuchsin stain than do the surroundings. Also in the third specimen, which is fixed in chrome, there are found heterotopic cortical islands. Staining by Sudan III and osmic acid reveals no or but little stainable lipoid content of the cells in the glomerular zone. The heterotopic islands of parenchyma aforementioned resemble the glomerular zone in this respect.

Diagnosis: Adrenal malformation and circulatory disturbances.

During the 5 months following the operation the boy increased 10 cm. in height and $4\frac{1}{2}$ kg. in weight (to 118 cm. and 26.2 kg., respectively). During the first months after the operation he was more calm and collected. Then again the sexual tendencies became more marked; he had daily erections and made sexual advances to adult women to the great perturbation of his parents. His temper also became worse. The 17-ketosteroids increased to 64 mg (Fig. 5), whereas the gonadotrophic hormone remained normal. The hair on the pubis grew as did the penis. His testicles remained the same except that the left testis which was the size of a small plum grew somewhat firmer. The prostatic gland had the size and consistency of that of a young man. The seminal vesiculae were not palpable. He suffered from facial acne. (Photo: Fig. 2).

It was suspected that there was a tumor of the left testis. This growth was removed Dec. 16, 1946.

Pathologist's report (Prof. H. Bergstrand).

»A round tumor, the size of a plum was found in the center of the testis, separated by a connective tissue capsule (Fig. 3). It is built similarly to the endocrine glands: it consists of groups of epithelial cells resembling follicles, separated by a network of capillaries. The epithelial cells are large due to abundant cytoplasm, irregular in shape and close to each other. The nuclei are large, mostly round and of varying size and chromatic content. Some are vacuolated, others richer in chromatin. With Mallory stain some of the cells show small red-colored granulae. Staining by Sudan III and osmic acid is negative as is the melanin stain of Masson. The adjacent testicular tissue is pushed to the side and does not show any signs of spermatogenesis — it is perfectly normal in appearance.

Diagnosis: Interstitial cell tumor.

The boy was followed 1 year after the last operation. The 17-ketosteroids output diminished (fig. 5). His rapid growth ceased im-

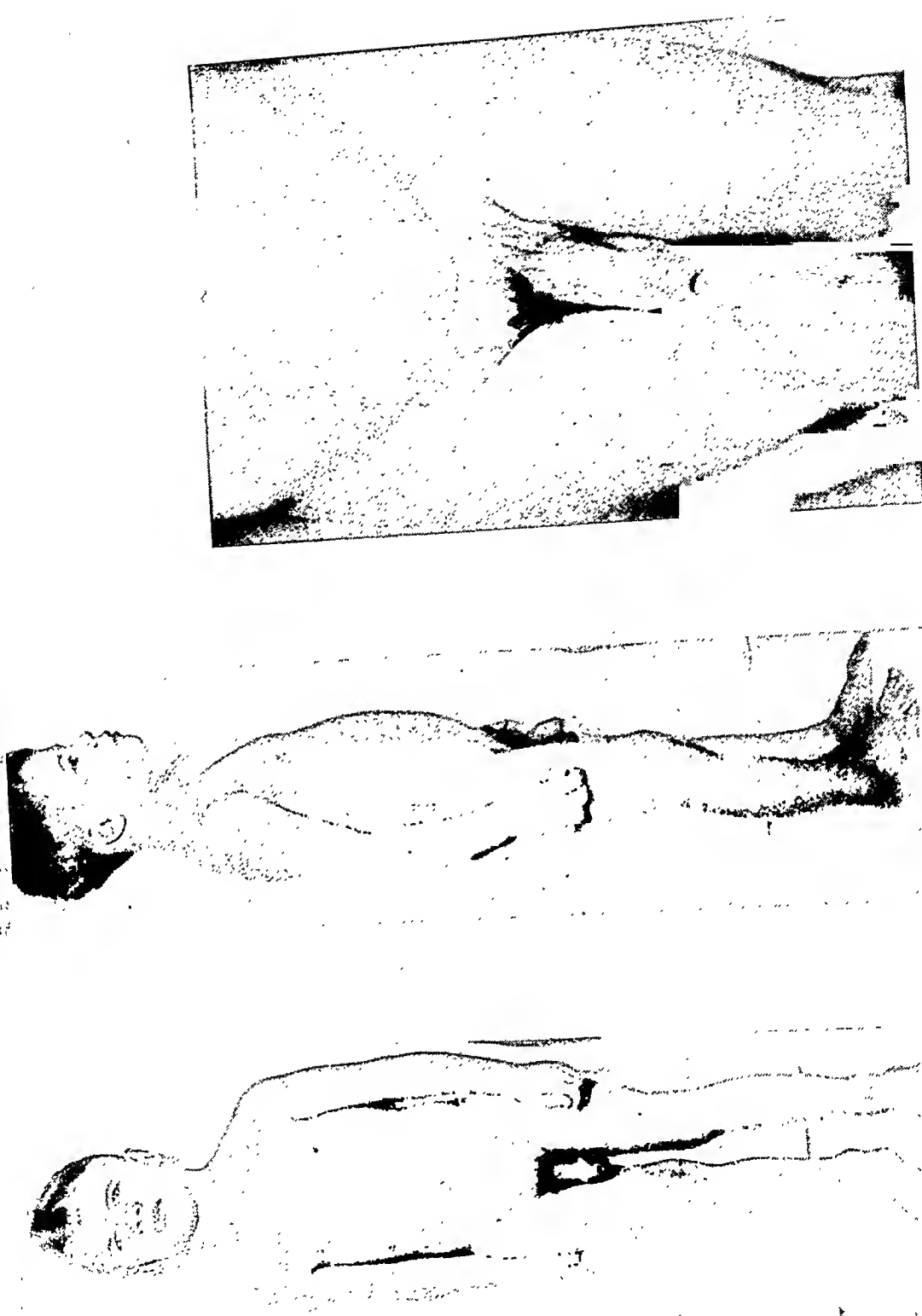


Fig. 2.

Patient at the age of 3 6/12 years. Note increased sexual and somatic development, with marked masculine appearance.

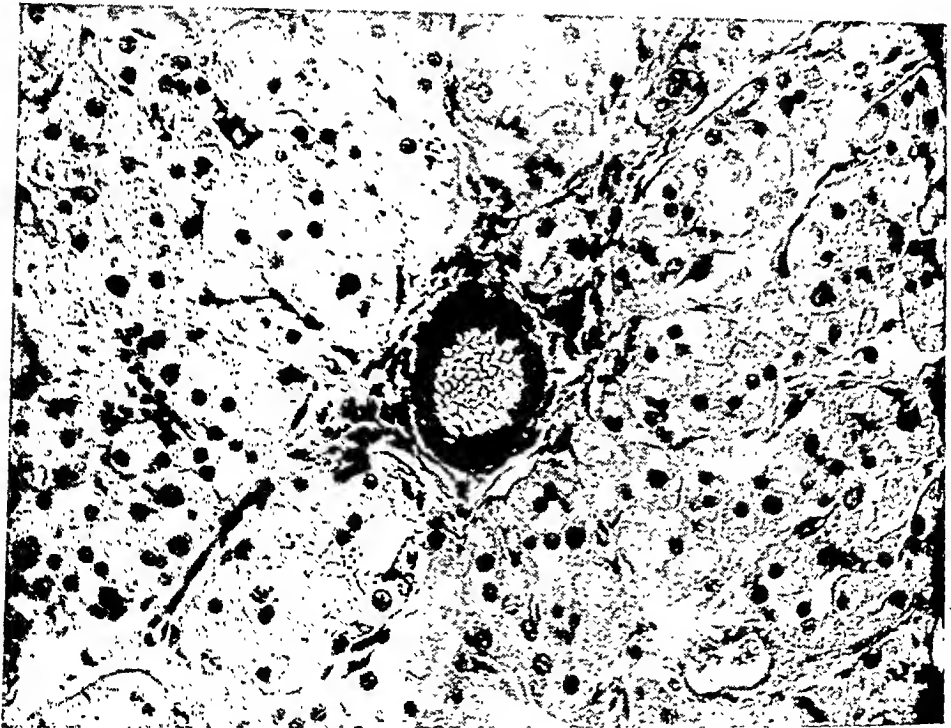


Fig. 3.

Cross section of the testicular tumor. Size indicated by the metric scale and $\times 450$ resp.

mediately. His height increased only 9 cm. and his weight 1.8 kg. His mental state has returned to the childish grade. He does not exhibit further sexual interest. Since the operation he has no further erections. After a few months the voice became lighter and he began to play with other children.

Physical examination Dec. 7, 1947. (Photo: Fig. 4).

The general build of the patient is more childish. The musculature is not so marked. The voice is still deep but lighter than previously. The facial skin is smooth and soft. The lips are not so protruding. The hair on the pubis is gone. The penis is 7 cm. long and 8 cm. in circumference. The glans is smaller than a year ago. The right testis is somewhat larger than previously. The prostatic gland is diminished and is now hardly palpable. Potassium in blood 17.2 mg %. The ketosteroid-output is 17 mg/24 hrs.

SUMMARY OF CASE REPORT.

A boy aged 2 11/12 years entered the hospital with a history of precocious development, especially of the sexual characteristics.

At examination his chronological age was 2 9/12 years, his intelligence age was 3 3/12 years, his skeletal age was 6 to 7 years and his sexual age was 17 years. He presented the clinical picture of isosexual precocity. It was, however, incomplete, as it later was shown that there was no spermatogenesis. The output of 17-ketosteroids was increased to values normal for a fullgrown man.

An abnormal left adrenal was removed. The operation was followed by a slight and transient regression of the symptoms.

The gonadotrophic hormone was normal but the 17-ketosteroids continued to increase. Five months later the left testicle was removed; it contained an interstitial cell tumor. Following this operation the symptoms immediately commenced to regress.

DIAGNOSIS

The different causes of precocious sexual development in boys are listed in the following table with the number of cases recorded in each group. (For further information see *Seckel*

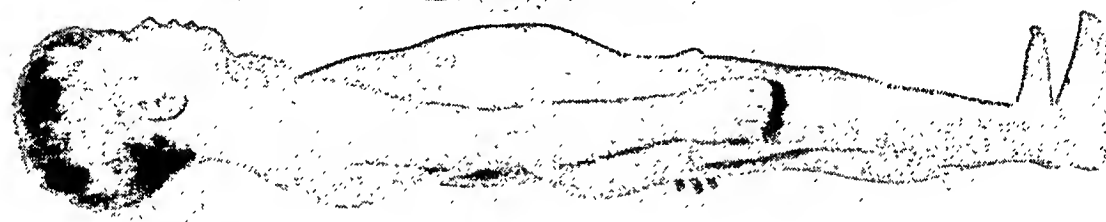


Table 1.
Precocious sexual development in boys.

Clinical Type and Pathology	No. of cases
I. Idiopathic Type (Without demonstrable pathology)	97
II. Cerebral Type	
a) Pineal neoplasms	21
b) Hypothalamic pathology	23
III. Adrenal-cortical Pathology	
a) Hyperplasia	5
b) Neoplasms	23
IV. Interstitial Cell Tumors of Testis	8

(1946), also Kaufmann (1907), Luft (1944) and Nation *et al.* (1944)).

One important difference between the two first groups on the one side and the two second groups on the other is, that the sexual precocity in the former is complete and in the latter is incomplete, i. e. in the former group the gonads are mature and in the latter group they are immature.

This is due to the fact that the former probably is caused by a supra-production of gonadotrophic hormone while in the latter the gonadotrophic hormones are normal for the age while the androgens are increased. The huge amount of 17-kelosteroids produced in this case proves it to belong to one of the two latter groups. It was also later shown to be a case of incomplete precocity with immature gonads.

The differentiation within this group between adrenal-cortical pathology and testicular neoplasm is dependent on the demonstration of tumor in either of these glands. In the present case, although no definite tumor was demonstrable, the difference in size of the testicles should have been strong evidence for a testicular tumor.

HORMONE STUDIES

This is the first case of this type in which the urinary androgens have been studied. (Fig. 5). (The hormone studies

were done by doctor *S. Mattson* of the Hormone Laboratory of the Sabbatsberg's Hospital). When first examined they were increased for the age (23 mg/24 hrs.) but still within the range of the normal adult male (*Talbot et al.*, 1942). Later they increased considerably (64 mg/24 hrs.). The gonadotrophic hormone was normal.

Venning (1942) made a study of the hormones in a group of men with an interstitial cell tumor. She found between 980 and 1040 mg. 17-ketosteroids per 24 hours. The inadequacy of the method used in both cases (*Zimmermann*) renders comparison uncertain. *Venning* considers her values to be 50 times greater than the normal for comparable ages. Referred to age the increase of 17-ketosteroids in the present case may be considered to be approximately the same (using as normal value for the age an output of 1—2 mg. per 24 hours as given amongst others by *Hamburger*, 1948).

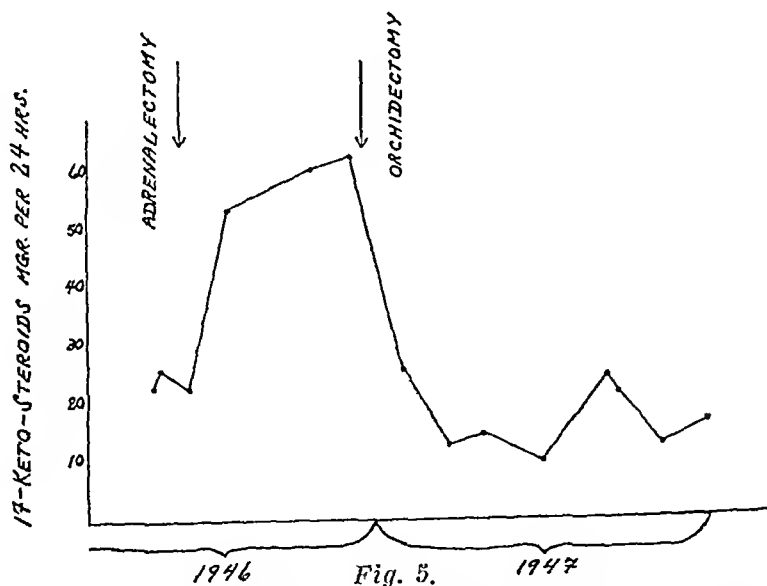


Fig. 5. 17-ketosteroids in mg per 24 hrs. urine during course of disease.

The increased output of urinary androgens in the present case was to be expected. It is further evidence of the fairly established fact that the interstitial cells of the testes by their production of androgens develop the secondary sexual characteristics in the male.

THE EFFECT OF THE OPERATION

Following the removal of the one adrenal a temporary regression of the symptoms was noted. The output of androgens was not influenced, however, and the amelioration can well be explained by the interference with the general condition caused by a major operation.

The effect of the removal of the interstitial cell tumor was striking.

An immediate check was put to the progress of all of the symptoms, including growth. A regression set in which was speedier and more pronounced in regard to the functional disorders and slower and less comprehensive in regard to the organic changes.

As shown by the graph the 17-ketosteroids returned to about one fifth of the maximum, that is to a value, normal for a 30-years old man.

The sexual activity stopped immediately and the personality regained its childish characteristics in about 3 months. As to the regression of the organic changes, the quality of the tissues was influenced rather than the size of the organs. The skin lost its coarseness and the acne disappeared, the subcutaneous tissue changed from manly firmness to childish softness, the lips became less protruding. The pubic hair diminished and was gone after 1 year, and the hair of the scalp became smoother. The musculature was less »herculean«. The prostatic gland also diminished.

On the other hand the voice remained deep and the penis remained the same size although it became softer.

For the sake of comparison the most striking aspects of the cases reported so far are presented in table 2.

It can be noted that the present case is the youngest on record both in regard to the age at the onset of symptoms and the age at operation.

Table

Reported cases of precocious sexual development

Author	Year of report	Age at onset	Age at operation	Age at report	Size of tumor
<i>Sacchi</i>	1895	5½	9½	10½	12×10 cm
<i>Rowlands et al.</i>	1929	6	9	11	5×4×3.5 cm
<i>Stewart et al.</i>	1936	4	5	6	1 cm Right testis twice the size of left
<i>Somerford</i>	1941	5	11	12½	10×7 cm
<i>Huffman</i>	1941	4	6	8	5 cm
<i>Urban</i>	1942	5	5½	6¾	4.5×3.5×2.5 cm
<i>Werner et al.</i>	1942	5½	6¾	7	2.5×1.5 cm
<i>Sandblom</i>	1948	2½	3½	4½	2.8×2.5 cm

2.

due to interstitial cell tumors of the testis.

Endocrine disturbance	Hormone studies	Effect of removal
Frequent erections, no ejaculations. Penis 9 cm. Beard 5 cm. 44 kg. 1.43 cm.	—	Beard disappeared. Penis 7½ cm. Right testis larger. No erections. Weaker. Stopped growing after 4 months. 6 months later no further change; right testicle slightly larger.
No ejaculations. Much hair on chest, loins and pubis.	—	No retrogression in signs of puberty. Hardly changed in any respect. Shaves regularly.
No hair in face. Pubic hair 5 cm. Voice slightly low. Penis 10 cm. Masculine interests but no abnormal behaviour.	Friedman test neg.	Pubic hair disappeared. Penis diminished to 8 cm. (Rem: Premature development of the seminiferous tubules in the testis where the tumor was. All the stages up to the formation of spermatozoa, but no formed spermatozoa.)
Pubic hair present. Shaving necessary since the age of 9.	—	No regression.
Moderate growth of pubic hair. Slight enlargement of both breasts. Genitals slightly larger than the normal.	Friedman test neg.	No regression.
Frequent erections. No ejaculations. No libido. Pubic hair. Voice broken.	Aschheim-Zondek test neg.	Pubic hair disappeared. No erections. Penis 1 cm. smaller.
Frequent erections. Facial and pubic hair. Voice deep. Taller, heavier, more muscular than normal. Penis 3.75 inches long, 1.5 inches in diameter. Libido had been manifested.	Friedman test neg.	Hair on upper lip disappeared, less genital hair. Voice the same. Aggressive attitude changed to gentleness. Left testicle increased.
Daily erections. Pubic hair. Voice deep basso. Penis 6 cm. Libido.	Aschheim-Zondek test neg. 17-ketosteroids 64 mg/24 hrs.	Pubic hair disappeared. No erections. More gentle and childish. Right testicle increased.

SUMMARY

A case of precocious sexual development due to an interstitial cell tumor of the testis is presented.

- 1: It is the eighth case reported.
- 2: It is the youngest case with an age of 2 6/12 years at the onset of the symptoms and 3 6/12 years at operation.
- 3: The 17-ketosteroids, prior to the operation as high as 64 mg. per 24 hr., decreased considerably some time following the operation.
- 4: After removal of the tumor the symptoms regressed — first the functional symptoms and then gradually the organic changes.

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ON THE QUANTITATIVE SPECTROPHOTOMETRIC DETERMINATION OF DEHYDROANDROSTERONE IN PURE SOLUTIONS*)

BY

AA. THEIL NIELSEN

Dehydroandrosterone occupies a position of importance in preparative chemistry, where it forms an intermediate in the synthesis of sex hormones, and also in the domain of physiological chemistry, where it is a vital substance in hormonal metabolism (see i. a. *Friedgood* (1944) and *Gallagher* (1944)). Nevertheless, to the best of our knowledge no specific method has hitherto been described for the quantitative determination of dehydroandrosterone. Below is an account of a simple method of determining the substance in pure solutions and of some questions relating to its specificity. An example of the clinical application of the method to the urine from a patient with a tumour of the adrenal cortex is published elsewhere (*Theil Nielsen, Pedersen-Bjergaard & Tonnesen* (1948)).

Kober (1931) found that when oestrone and certain other naturally occurring oestrogens are heated with phenolsulfonic acid and then diluted with water or suitably diluted sulfuric

*) Presented for the Danish Society for Endocrinology, January 7, 1948.

acid, they give a red solution. *Dirscherl & Zilliken* (1943) examined the response of other steroids to the same treatment, but using sulphuric acid instead of phenolsulphonic acid. They found that when heated with sulphuric acid, dehydroandrosterone gives a yellow solution. If about an equal volume of water is poured into it, a blue-violet ring appears at the interface. When the fluids are mixed we get a clear solution of the same colour. These workers suggest the application of the reaction to quantitative purpose, but they describe no method for it. In the following an endeavour has been made to examine the factors (heating time, acid concentrations, etc.) that have a bearing on the quantitative course of the reaction.

EXPERIMENTAL

1. Spectral composition of the reaction colour.

0.4 ml of a 50 mg % absolute-alcoholic solution of dehydroandrosterone was transferred by pipette to a testtube (20×180 mm.), which was then placed in a beaker with iced water; 2.00 ml. sulphuric acid was then added. After stirring thoroughly with a glass rod the tube was placed into a boiling water-bath for 90 seconds. The contents were stirred at the beginning and end of the heating process. The tube was then replaced in iced water. The coloured reaction mixture was diluted with 8.00 ml. 25 vol. % sulphuric acid (1 vol. sulphuric acid diluted with water to 4 vol.). After stirring thoroughly the extinction coefficient was determined in the range 400—700 m μ with intervals of 10 m μ . Measurements were made with a Beckman quartz-spectrophotometer, model DU. For comparison an experiment was made by diluting with 100 % instead of 25 % sulphuric acid, for the purpose of obtaining an idea of the colour formation immediately after the initial heating. Fig. 1 is a graphic representation of these two experiments. It will be seen that the characteristic colour has its absolute absorption maximum at 600 m μ .

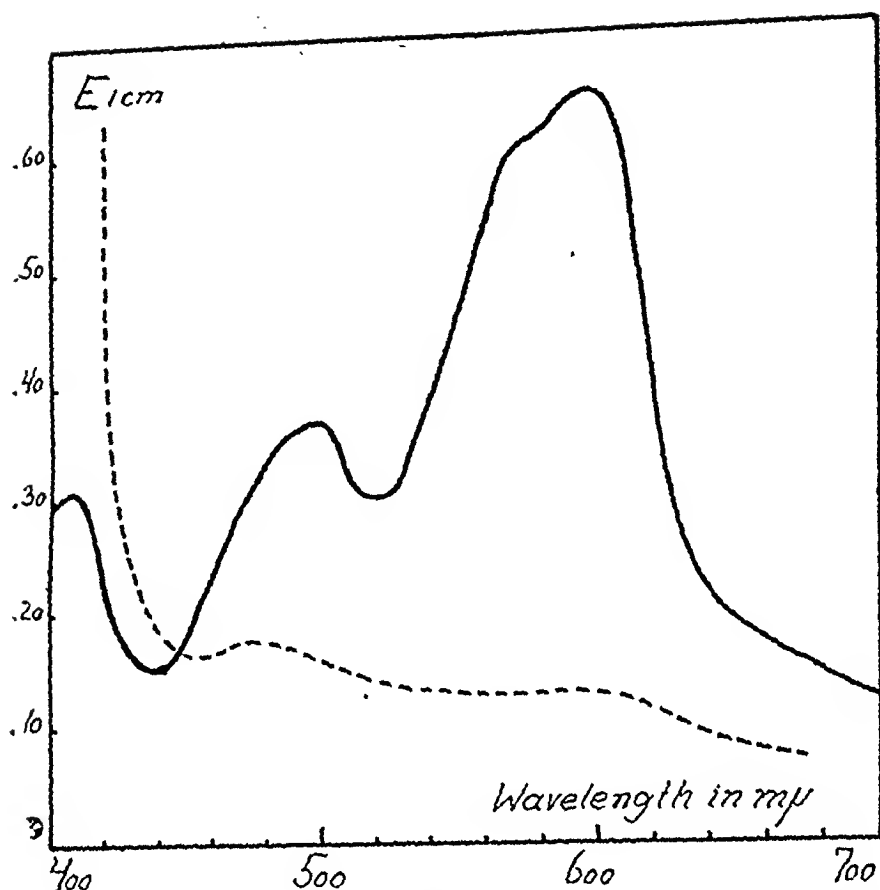


Fig. 1.

Absorption spectra for 0.2 mg. dehydroandrosterone after sulphuric acid reaction.

Abscissa: Wave length in $m\mu$.

Ordinate: Extinction coefficient.

————: Dilution with 25 vol. % sulphuric acid.

-----: Dilution with conc. sulphuric acid.

2. Heating time.

The same experiment as under 1., but with the difference that the heating time (boiling waterbath) was varied from 0.25 to 10 minutes. After diluting with 25 vol. % sulphuric acid the extinction was determined at 600 $m\mu$. The result will be seen from figure 2, which shows that the maximum colour intensity appeared after 1.5 min. heating, and that if the reaction is to be reproducible this time must be strictly adhered to. This is the reason why the tubes were placed in iced water before and after heating, as described above.

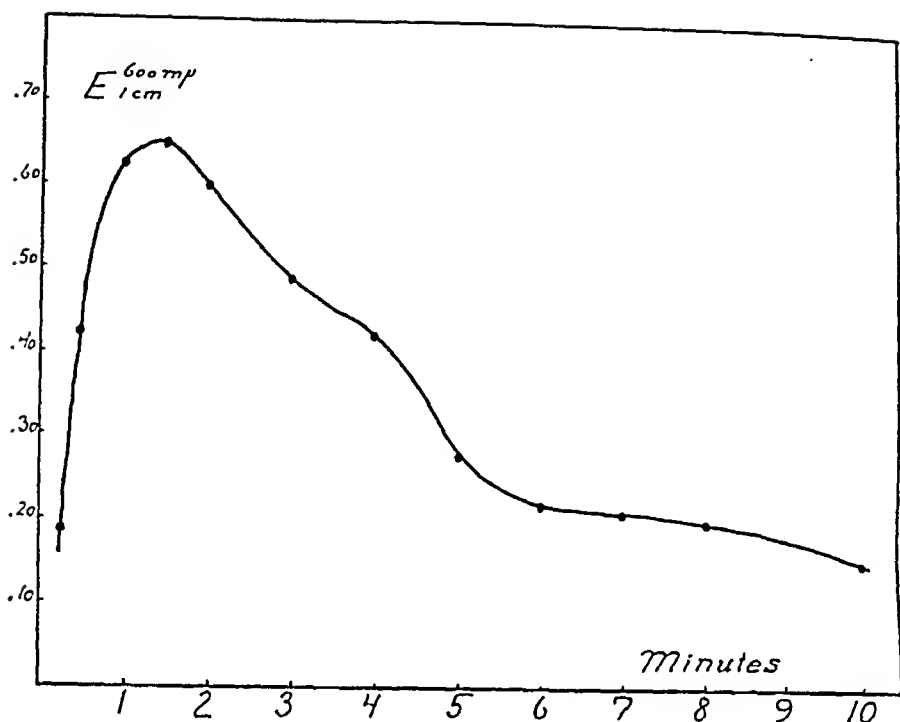


Fig. 2.

Dependence of colour intensity on heating time.

Abscissa: Heating time in minutes.

Ordinate: Extinction coefficient at 600 $m\mu$.

3. Acid concentration in the final dilution.

A series of experiments was set up with 0.2 mg dehydroandrosterone, proceeding as under 1, except that after heating, we diluted with sulphuric acid of concentrations varying from 0 to 100 %. Fig. 3 shows the extinction coefficients (600 $m\mu$) plotted against the sulphuric acid concentration (vol. %) in the finally diluted reaction mixture.

It will be seen that of the acid concentrations employed the 50 % gives the most intense colour. But if the acid concentration is varied, we get not only quantitative but also qualitative changes in the colour composition. Fig. 1 already shows the absorption spectra of reaction mixtures containing 40 and 100 % sulphuric acid. It will be seen that there is a great difference between them, quite apart from the intensity

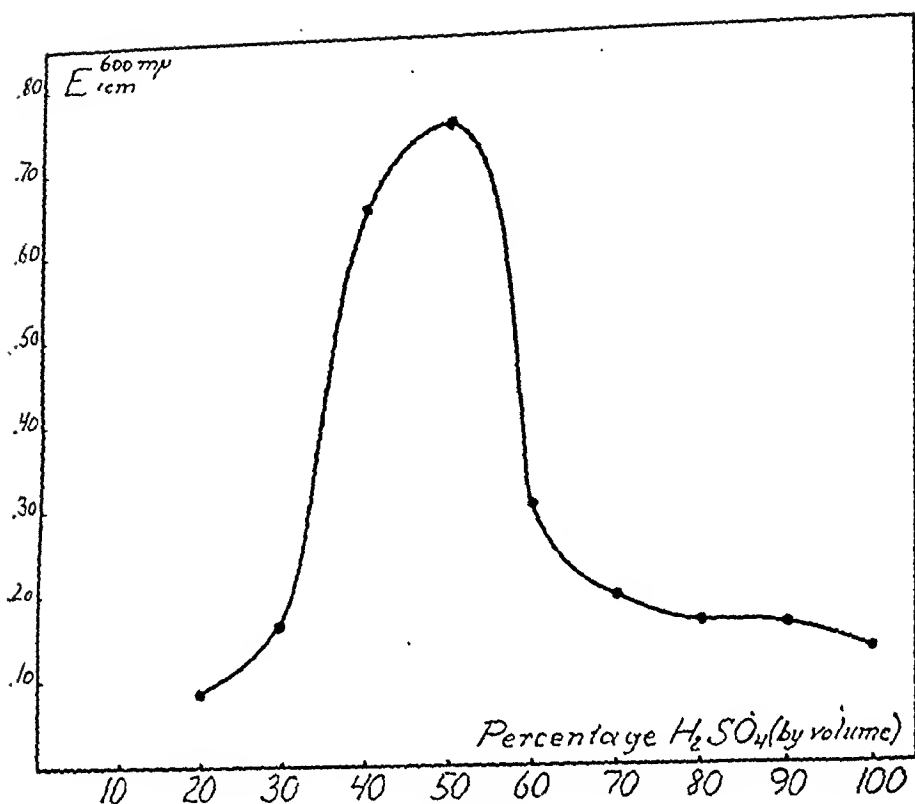


Fig. 3.

Influence of acid concentration on colour development.

Abscissa: Concentration of sulphuric acid (vol. %) in the final reaction mixture.

Ordinate: Extinction coefficient at 600 m μ .

at 600 m μ . The same appears from fig. 4, though less pronouncedly; it contains absorption spectra for reaction mixtures with 40, 50, and 60 vol. % sulphuric acid. This difference can also be seen with the naked eye: the mixture containing 40 % sulphuric acid is blue-violet, with 50 % pure blue, and with 60 % blue-green.

Judging from the above, a content of 50 % sulphuric acid in the final mixture is preferable (i. e. dilution should proceed with 8 ml. of a 37.5 vol. % sulphuric acid). In actual fact, however, matters are more complicated, as will appear from the following:

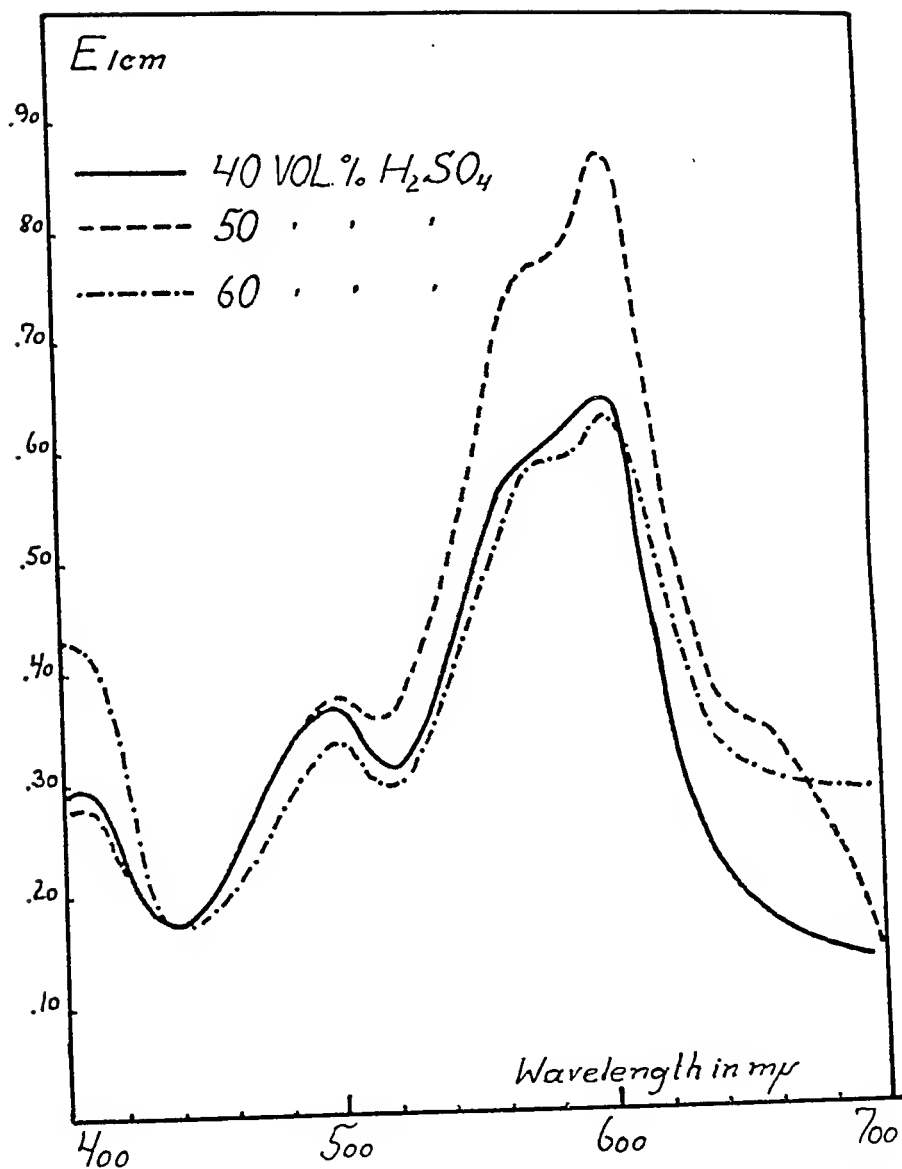


Fig. 4.

Absorption spectra of the reaction colours in mixtures with 40, 50, and 60 vol. % sulphuric acid.
 Abscissa: Wave lengths in $m\mu$.
 Ordinates: Extinction coefficients.

4. Stability of the reaction colour.

This was tested for reaction mixtures containing 40 and 50 vol. % sulphuric acid. The extinction was measured at

600 m μ every 10 minutes up to an hour after diluting. The result is shown in fig. 5.

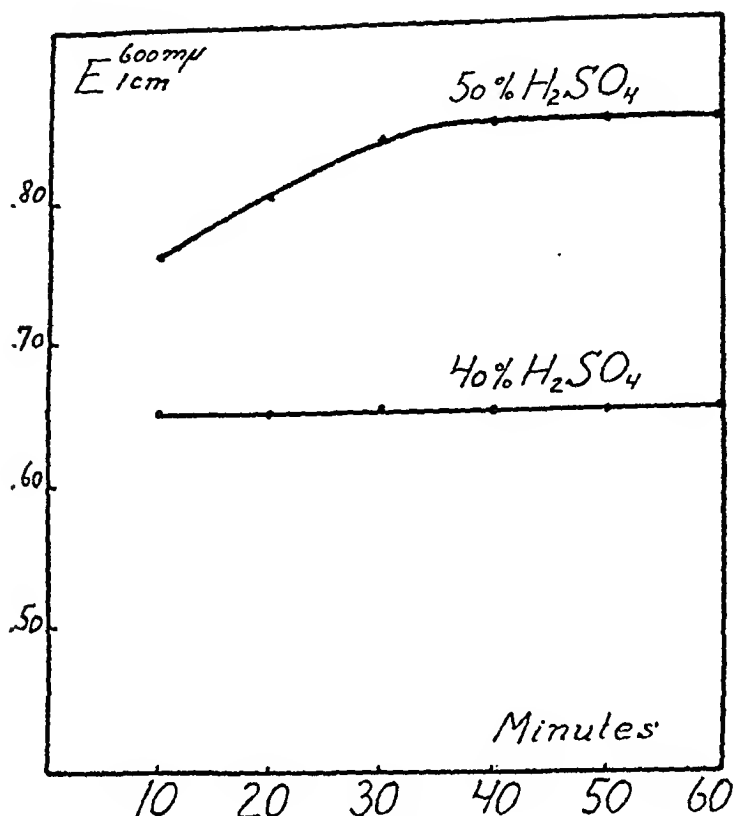


Fig. 5.

Stability of reaction colour for mixtures containing 40 and 50 vol. % sulphuric acid.

Abscissa: Time in minutes after final dilution.

Ordinate: Extinction coefficients at 600 m μ .

The experiments showed that with 40 vol. % acid we obtain a stable colour almost at once, whereas with 50 vol. % acid it is only stable after about 40 minutes. In any case the reaction colours are stable for four hours. After a longer time there will be turbidity, with a precipitation of a blue pigment.

After these experiments there is evidently a choice between a more sensitive reaction which cannot be read until after 30 to 40 minutes, and a slightly less sensitive one which can be read immediately. We chose the latter, as in the practical

applications of the method hitherto there have been such quantities of dehydroandrosterone available that somewhat less sensitivity was of minor importance.

5. *Validity of Lambert-Beer's law.*

Experiments with dehydroandrosterone varying in quantity from 0 to 0.300 mg. have shown downright proportionality between the quantity worked with and the extinction coefficient at 600 m μ (fig. 6). Larger quantities were not tested, as they involve technical difficulties and are of no practical interest.

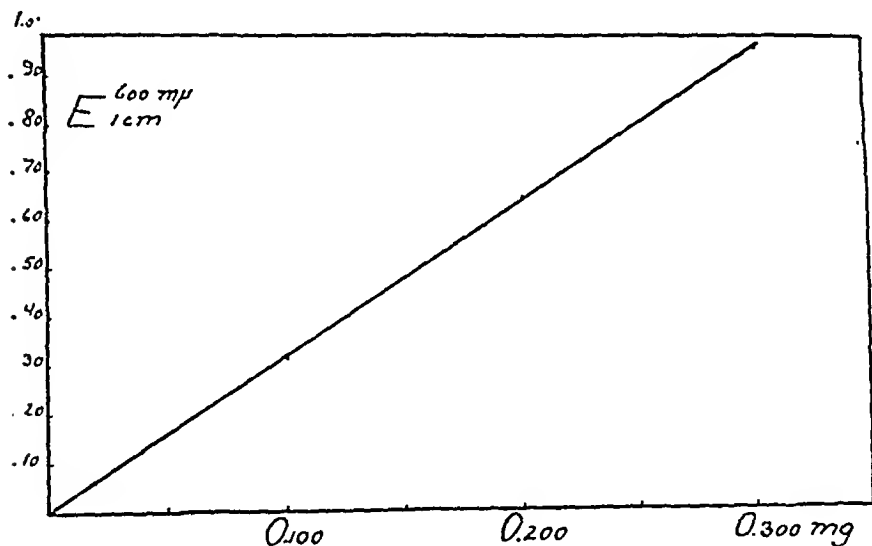


Fig. 6.

Linear dependence between quantity of dehydroandrosterone and extinction.

Abscissa: mg. dehydroandrosterone.

Ordinate: Extinction coefficients at 600 m μ

6. *Accuracy of the method.*

As the investigation in its present form aimed solely at determining dehydroandrosterone in pure solutions, the question of accuracy for the time being may be reduced to the fol-

lowing: With what certainty can the colour reaction be reproduced, given the same quantity of dehydroandrosterone? We have not examined the point systematically, but only in so far as the above experiments have provided the occasion for it. If we disregard the errors ($< 5\%$) that are associated with pipetting the small volumes of fluid, reproducibility for the most part depends upon the exact observation of the heating time. Small variations in this time have given rise to deviations of 5 to 10 % from the average. These are compensated for by including a dehydroandrosterone standard of 0.200 mg. Thus the uncertainty found by experience may be put at a maximum of 5 %.

7. Specificity of reaction.

While referring the reader to the discussion below, we shall give a brief account of the reaction colours which, under the experimental conditions described above, are obtained with certain steroids more or less closely related to dehydroandrosterone, chemically or in their occurrence. Tests were made with 0.2 mg. of the following:

- a. Androsterone (Androstane-3 α -ol-17-one);
- b. Isoandrosterone (Androstane-3 β -ol-17-one);
- c. Δ^4 -Androstene-3,17-dione;
- d. Δ^5 -Androstene-3 β ,17-diol;

The absorption spectra for these substances are shown in fig. 7.

It will be seen that of the substances tested, the only one giving a reaction resembling that of dehydroandrosterone is androstenedione. It must be observed, however, that the colour is very easy to distinguish from that of dehydroandrosterone, being very unstable when heated for a few minutes, whereas the reaction colour with dehydroandrosterone will tolerate that treatment.

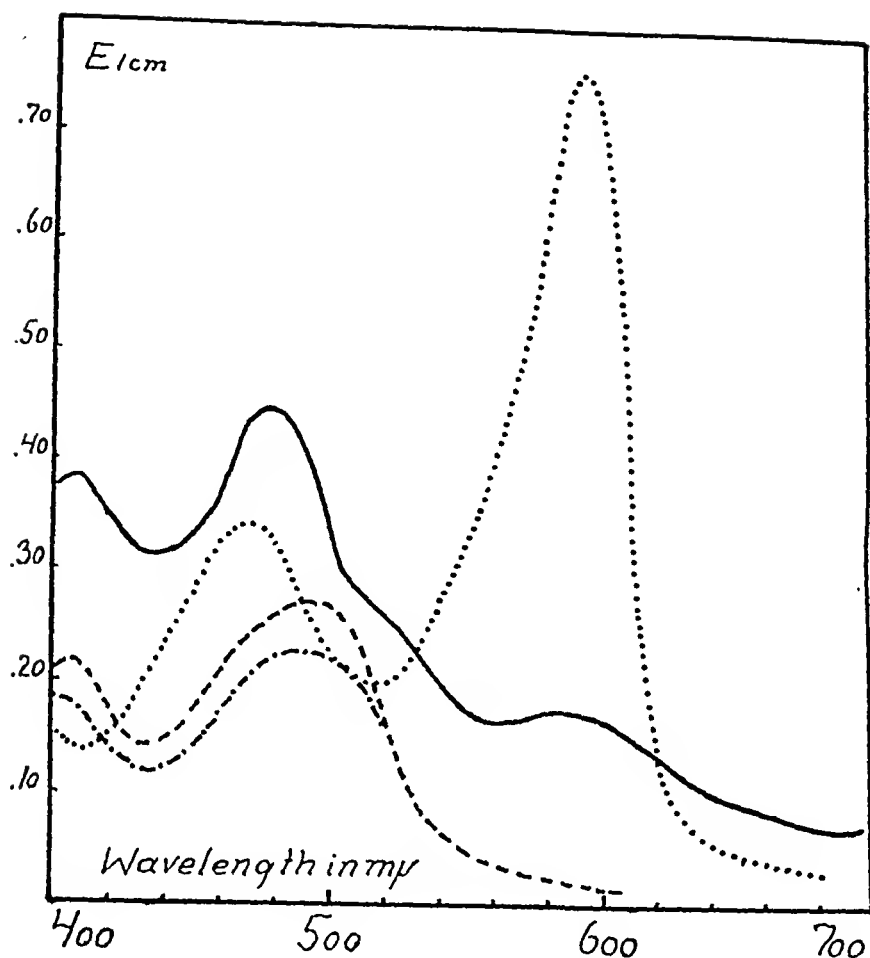


Fig. 7.

Absorption spectra for 0.2 mg of the following substances in the sulphuric acid test:

Abscissa: Wave length in mμ.

Ordinate: Extinction coefficients.

- : Androstenediol;
: Androstenedione;
 -----: Isoandrosterone;
 - · - · - ·: Androsterone.

CONCLUSION

The analytical method worked out in the foregoing may briefly be described as follows: Of the sample, prepare an absolute-alcoholic solution about 0.5 mg dehydroandrosterone

per ml. From this solution pipette 0.4 ml. into a test tube (20×180 mm.). Place the test tube in iced water, add 2.0 ml. sulphuric acid and then heat while stirring in a boiling waterbath for 90 seconds. Replace the tube in iced water. After diluting with 8.0 ml. 25 vol. % or 37.5 vol. % sulphuric acid, determine the extinction at 600 m μ (10 mm. cells). In the former case the result can be read immediately after dilution, in the latter after 40 minutes.

At each determination include a dehydroandrosterone standard of 0.200 mg.

DISCUSSION

Dirscherl & Zilliken's colour reaction for dehydroandrosterone can be adapted without difficulty to quantitative analysis. The experimental conditions which have had to be standardized are the same as those encountered in many similar reactions, and the treatment of this point presents nothing that is particularly new.

On the other hand, a significant problem involved by the application of the method in physiological and endocrinological chemistry is the specificity of the reaction. *Dirscherl & Zilliken* state that the following substances react negatively: Androsterone, testosterone, androstenedione, oestrone, progesterone, corticosterone and isoandrosterane-17-ol-6-one. The only substance they found with a positive reaction was isoandrosterane-6-ol-17-one.

Of the substances which we have examined — apart from dehydroandrosterone — most interest attaches to isoandrosterone. It reacts negatively to the sulphuric acid test, a fact that is of decisive importance to its use for endocrinological and similar purposes. Like dehydroandrosterone, isoandrosterone is a β -steroid, and therefore the two substances cannot be separated by digitonin precipitation, which otherwise is a method often used for the analysis of ketosteroid mixtures from urine and other organic material (see e. g. *Bau-*

mann & Metzger (1940) and *Frame* (1944)). Nor has separation been possible by chromatographic adsorption analysis (*Dingemanse et al.* (1946)).

Gallagher (1944) has reported briefly on some experiments with a Pettenkofer reaction modified by *Kerr & Hoehn* (1944), a reaction said to be fairly specific to dehydroandrosterone. This reaction does not seem to be so simple either in procedure or as regards reagents as the sulphuric acid reaction. And, as far as we are aware, no quantitative adaption has yet been published.

SUMMARY

Dirscherl & Zilliken's colour reaction for dehydroandrosterone is adapted to a quantitative spectrophotometric method of determination. Certain factors having a bearing upon the course of the reaction are examined. It is shown that isoandrosterone occurring together with dehydroandrosterone in urine, not being separable from dehydroandrosterone by the methods hitherto described, gives no reaction.

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THE EFFECT OF X-RAY IRRADIATION ON THE
PITUITARY BODY OF INFANTILE RATS
TREATED WITH α -OESTRADIOL
MONOBENZOATE

BY

A. BAIDINS, L. CLAESSION AND A. WESTMAN

Numerous investigations have been published dealing with the influence of X-ray and radium irradiations on the ovarian function. Both the effect of the direct irradiation of the ovaries and the influence of the X-ray irradiation of the pituitary body on its gonadotrophic hormone production were thoroughly investigated. However, the results of the latter are not unanimous as it may be seen from the data collected by *Denniston* (1942) and from our recently published survey of the corresponding literature (*Baidins, Claesson & Westman*, 1946). Some authors, among others *Savasaki* (1933) considered that premature oestrus could be elicited by weak irradiation of the pituitary body, while on the other hand others were unable to confirm his results. In one of our experiments it has been found that the irradiation of the pituitary body with X-ray doses from 2 to 45 r. did not damage to an appreciable extent the ovaries of infantile rats neither in function nor in anatomical structure. We have found further that even much higher doses i. e. 500—1000 r. did not produce any noticeable disturbance of the ovarian function.

On the other hand it is well known from clinical experiences that by using X-ray doses of the same magnitude of order the function of the pituitary gland can be influenced and its

gonadotrophic hormone production stimulated. The results of the experiments reported above are not in accordance with the clinical observations and are therefore rather surprising; but it must be taken into account that there are principal differences in the reaction of laboratory animals and human beings. Furthermore it must be emphasized that in nearly every case of therapeutic X-ray treatment pathologically altered and not quite normally functioning pituitary bodies are irradiated. Therefore it may be assumed that pathologically changed pituitary glands are more sensitive to the action of X-rays than the normal ones.

For the further elucidation of this both theoretically and practically important question experiments were carried out by us on juvenile rats treated with oestradiol*) before X-ray irradiation (*Baidins, Claesson & Westman* 1948). In the animals, treated in this way, the pituitary body undergoes typical histological changes, which are well known in their characteristic features from the studies of numerous earlier investigators. It needs here only be recalled that the anterior lobe increases in size due to the increase in the number of the cells. Both the eosinophilic and the basophilic cells show signs of degranulation and their relative number decreases according to the increase of the chromophobe cells. Treatment with oestradiol causes a depression in the gonadotrophic hormone production of the pituitary body.

In our experiments referred to above a series of juvenile female rats were treated with oestradiol while an other series of animals were treated with oestradiol combined with X-ray irradiation. The doses administered were 600 and 2000 r. respectively. There was a characteristic difference between these two series; the ovaries of animals treated only with oestradiol were small with an average weight of 32,9 mg. By the examination of these ovaries only a very small number of mature follicles and newly formed corpora lutea were identifiable, and only a weak degree of luteinization was found. On the other hand the ovaries of the animals treated

*) In the text »oestradiol« means oestradiol monobenzoate.

with oestradiol combined with X-rays were large, their average weight being 59,3 mg. after the administration of 600 r. and 52,5 mg. after the administration of 2000 r. The follicles were well developed and large, newly formed corpora lutea were present.

Apart from a more expressed hyperemia of the pituitary body found in animals treated with X-rays combined with oestradiol, the microscopical appearances of the pituitary bodies in both groups were alike.

Hence, these our studies revealed the interesting fact that X-ray irradiation of the pituitary body does not produce any identifiable changes either in the histological appearance or in the function of the ovaries, whereas considerable changes could be observed in the ovaries after the X-ray irradiation of pituitary glands altered by previous treatment with oestradiol.

It seemed to us of interest to examine the question whether these changes in the response of the pituitary body to irradiation occur also in infantile animals, or are they restricted to the age of puberty and maturity.

EXPERIMENTAL

The present study was made on 25 female rats aged 8 days; of these 11 served as controls. Every control was of the same litter as the corresponding experimental animal. The vaginal smear of the animals was daily examined and their weight controlled on every third day. 200 I. B. U. oestradiol monobenzoate was administered subcutaneously to 14 experimental animals every day over a period of 8 days: the total amount administered was 1600 I. B. U. After this previous treatment the animals were irradiated with a single dose of 40 r. The 11 control animals were divided into 2 groups; in the first group 7 of the 11 animals were treated with oestradiol in the very same way as the irradiated ones, in order to study the reaction of the pituitary body to estradiol treatment. In the second group the remaining 4 animals did not get any treatment at all.

Before irradiation the animals were fixed in dorsal position and with the exception of a small area of the head, protected with lead plates. We are indebted to *Dr. A. R. Forsberg*, member of the Staff of the Radiopathological Department of the Radiumhemmet of Stockholm for the measurements of the dosage. Irradiation was performed with the use of a stabilovolt apparatus and metallix tubes: the conditions were 160 kv. 7. ma., 0.5 mm. Cu and 6.23 r/m.

After the irradiation the animals were observed over a period of 50 days and then sacrificed. The pituitary bodies were immediately weighed, fixed in Susa (Heidenhain) and embedded in paraffin. Serial sections were made, each sections measuring $4\ \mu$ in thickness, and the sections stained with Azan according to Heidenhain's description. The ovaries were weighed, and the sections stained with hematoxylin — eosin. The vagina and uterus were histologically also examined.

During this observation time the animals did not manifest any signs of disturbances attributable to the injurious effect of either oestradiol or X-rays. Their weight-curves were normal.

The effect of oestradiol treatment manifested in an immediate oestrus reaction in the vagina. Later this oestrus ceased, but reappeared when the animals reached the age of puberty. This oestrus reaction took place in both oestradiol treated and oestradiol treated + irradiated animals in the 5th week of their life, while in the untreated controls spontaneous oestrus did not occur before the 6th week. The cyclic processes were normal, excepting three cases of irradiated animals showing no typical cornified cells.

Pituitary body. The pituitary bodies of the animals treated with X-rays combined with oestradiol showed the characteristic changes which, as mentioned above, follow oestradiol treatment. As it is shown in Table 1, there was a slight increase in weight due to the increase in size of the anterior lobe. The number of the chromophil cells decreased while that of the chromophobes increased. Both the eosinophiles and basophiles showed degranulation.

Table 1.

Average Weight of the Pituitary Body and the Ovaries of Infantile Rats treated with Oestradiol and X-rays.

(The animals were examined 58 days after the first injection and 51 days after irradiation).

Material	Number of Animals	Average Weight of Pituitary body in mg.	Average Weight of Ovary in mg.
Irradiated animals previously treated with oestradiol	14	7.0	35.0
Controls treated with oestradiol	7	6.6	26.1
Untreated controls	4	5.2	27.5

X-ray dose: 40 r. Oestradiol: 1600 I. B. U.

Apart from a slightly higher degree of hyperaemia of pituitary glands found in animals treated with X-rays combined with oestradiol, the microscopical appearances did not show any other difference in these two groups.



Fig. 1.

Ovary of an Infantile Rat treated with a total Dose of 1600 I. B. U. Oestradiol over 8 days. $\times 28$.

Ovaries. The ovaries of the animals treated with oestradiol weighed about the same as those of the untreated controls. The number of the Graafian follicles was reduced and large, intensively vascularized corpora lutea were in an increased number present (Fig. 1). The ovaries of the irradiated animals treated with oestradiol differed considerably from those of the non-irradiated ones. The ovarian weights in this group

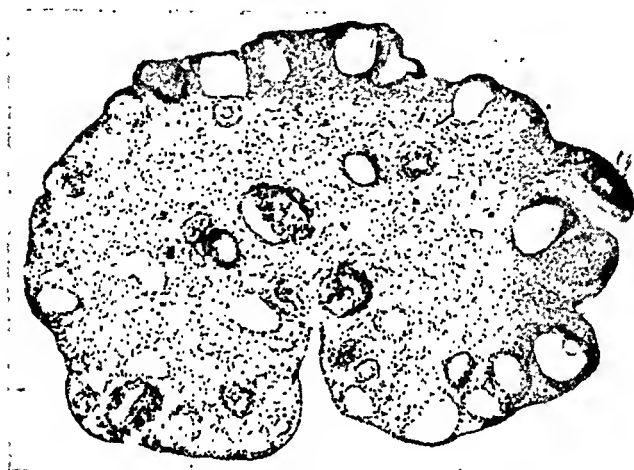


Fig. 2.

Effect of X-ray Irradiation of the Pituitary Body on the Ovary of Infantile Rat after Previous Oestradiol Treatment. $\times 28$.

were significantly higher as shown in Table 1. No Graafian follicles were present and some of the growing follicles showed atretia. On the other hand a well developed interstitial gland was found. In this group the corpora lutea were entirely absent in 5 of the 14 animals and the ovarian parenchyma was to a large extent constituted by interstitial gland (Fig. 2). In the ovaries of the remaining 9 animals of this group both the interstitial gland and the corpora lutea were to be found.

DISCUSSION

The present investigation and the results reported in our earlier communication (*Baidins, Claesson & Westman, 1948*) have shown that in infantile rats the X-ray irradiation of the

normal pituitary body with a single dose of 40 r. did not affect to an appreciable extent its production of gonadotrophic hormone. Hence, our studies have not brought forward evidence in supporting the assumption that X-ray irradiation of a normal rat pituitary could stimulate its production of gonadotrophic hormone and could thereby produce premature oestrus. X-ray irradiation combined with oestradiol treatment, however, yields different results. The ovaries increase in size and weight as compared to those of the corresponding controls. This increase in size and weight depends chiefly upon the intensive development of the interstitial gland. At the same time a marked degeneration of the follicles could be observed. The infantile animals show quite another reaction than the juvenile ones. The reason for this phenomenon may be cleared up by further investigations on the function of the interstitial gland under the given experimental conditions.

Some experiments of this type are in progress at this Institute.

SUMMARY

1. X-ray irradiation of the pituitary bodies of normal infantile rats in doses between 2—1000 r. does not influence their gonadotrophic hormone production.
2. Oestradiol treatment of normal infantile rats did not produce any changes in the gonadotrophic function of the pituitary gland examined two months after the treatment. The ovaries of these animals have normal weight and normal histological appearance.
3. X-ray irradiation of the pituitary bodies of normal infantile rats sensitized by previous oestradiol treatment affects the function of the pituitary gland; two months after the combined treatment the follicles show degenerative changes, and the ovaries are significantly increased in weight and size due to the intensive development of the interstitial gland.
4. The histological appearance of the pituitary bodies in the treated and non treated animals was alike.

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A SPECTROPHOTOMETRICAL INVESTIGATION
OF THE EXCRETION OF DEHYDROANDRO-
STERONE IN THE URINE OF A WOMAN
WITH A VIRILIZING ADENOMA OF
THE ADRENAL CORTEX

BY

AA. THEIL NIELSEN, K. PEDERSEN-BJERGAARD &
M. TØNNESEN

It is a familiar occurrence when virilizing tumours of the adrenal cortex are accompanied by an abnormally high excretion of 17-ketosteroids in the urine. The literature on the subject, a considerable part of which is cited by *Parkes* (1945), is already voluminous. An increased excretion of this kind has long been regarded as a diagnostically valuable indication of the presence of a tumour in the adrenal cortex. *Callow & Crooke* (1944) are among those authors who have drawn attention to the fact that in certain cases this belief may lead to erroneous conclusions. Increased excretion of 17-ketosteroids is not confined solely to tumour cases, for it also occurs in simple hyperplasia of the adrenal cortex. Thus it has been impossible definitely to differentiate between the two affections on the basis of the absolute values of the 17-ketosteroid excretion alone. On the other hand, it is held that fractionating the 17-ketosteroids into α - and β -ketosteroids, of which the latter can be precipitated with digitonin, but not

the former, is capable of giving valuable information. In a case of hyperplasia of the adrenal cortex they find that even if the total excretion is increased (79—100 mg. in 24 hours), the ratio between the β - and α -forms are almost the same as that found by *Talbot et al.* (1940) in normal men, women and children, viz. about 1 to 10. Among tumour patients, however, *Callow & Crooke* find a marked increase in this ratio. They give no figures of their own, but refer to *Talbot et al.*'s work cited above, which finds that in cases of adrenal cortical tumours the β -compounds may represent 50 to 60 % of the total 17-ketosteroids. *Callow & Crooke* state the conclusion of their investigations as follows: »It is suggested that the isolation of dehydro(iso)androsterone from urine or the finding of a high ratio of β - to α -ketosteroids in urine is of fundamental importance in the diagnosis of adrenal cortical tumours«. *Friedgood* (1944) discusses a similar theory. He too is unable to find a definite difference between the total 17-ketosteroid excretion by women with adrenal cortical tumours and those with hyperplasia; but, like *Callow & Crooke*, he finds that in tumour cases the β -ketosteroid amounts to 30 to 70 % of the total 17-ketosteroids, whereas in hyperplasia the highest is 25 %. However, *Friedgood* is very reserved as regards the conclusions to be drawn from a material so small as that hitherto examined.

Although much is still unclarified, there is no doubt that the more qualitative investigation of the excretion of 17-ketosteroids referred to below represents progress. It must be realized, however, that fractionating on the basis of digitonin precipitation is somewhat rough. Each of the fractions, α - and β -, consists of many substances, and therefore it would be of advantage to have specific methods for the quantitative determination of the various ketosteroids. In cases like the present, what interests us is especially the substances of the β -fraction, of which dehydroandrosterone and isoandrosterone occur most frequently and in the greatest quantity. To our knowledge no method has previously been described for the purpose, and therefore in the following we propose to give an

account of some experiments made on the efficacy of the *Theil Nielsen* (1948) method published from this laboratory for the quantitative determination of dehydroandrosterone, a method that is based upon a colour reaction published by *Dirscherl & Zilliken* (1943).

The experiments were made with urine from a woman with a bilateral adrenal cortical *adenoma* (as for the case record, see: *Leth Pedersen*, 1948). The urine has previously been analyzed in this laboratory (*Theil Nielsen, Pedersen-Bjergaard & Tønnesen*, 1947). The paper having been published in Danish it will be summarized very briefly here: The patient, a woman 45 years old, for about twenty years has suffered from increasing virilization (hirsutism, growth of beard, baldness, failing sexual function, etc.). The biological androgenous excretion was then found to be very high, about 300 capon units in 24 hours. The excretion of 17-ketosteroids was correspondingly increased, about 600 mg in 24 hours. It was supposed from a comparison of the biological and photometric analyses that the patient excreted chiefly dehydroandrosterone, a result that was confirmed by the isolation of considerable amounts of crystalline dehydroandrosterone from the urine.

The above analyses were carried out on urine before the laparotomy. The experiments described below, however, were made on a sample of urine collected *after* the operation. In that operation the tumour was removed, which perhaps is the reason why the excretion of 17-ketosteroids decreased, as will be seen by a comparison with the following experiments.

EXPERIMENTAL

A. Collection and extraction of urine. Ten days' full diuresis, amounting to 4.5 l. was received from the hospital. The urine was extracted as described by *Dingemanse & Laqueur* (1938). The extract was dissolved in 100 ml. absolute alcohol, and this solution is described in the following as the »crude extract«.

B. Photometric analysis: The total content of 17-ketosteroids was determined by means of *Callow, Callow & Emmens'* (1938) modification of the m-dinitrobenzene reaction. The

total crude extract was found to contain 1000 mg. 17-ketosteroid, corresponding to a *daily excretion of 100 mg.*

The possibility of the direct application of the aforementioned modification of *Dirscherl & Zilliken's* colour reaction to dehydroandrosterone was then tested: In a test tube 0.02 ml crude extract was diluted to 0.4 ml. with absolute alcohol, and this solution was treated as described by *Theil Nielsen*. The resulting reaction mixture was examined spectrophotometrically in the range 400—700 m μ . The apparatus used for all readings was a Beckman quartz-spectrophotometer, Model DU. Absorption readings were taken with intervals of 5—10 m μ with the use of 10 mm. cells. The absorption spectrum together with a corresponding curve for 0.2 mg dehydroandrosterone is shown in fig. 1.

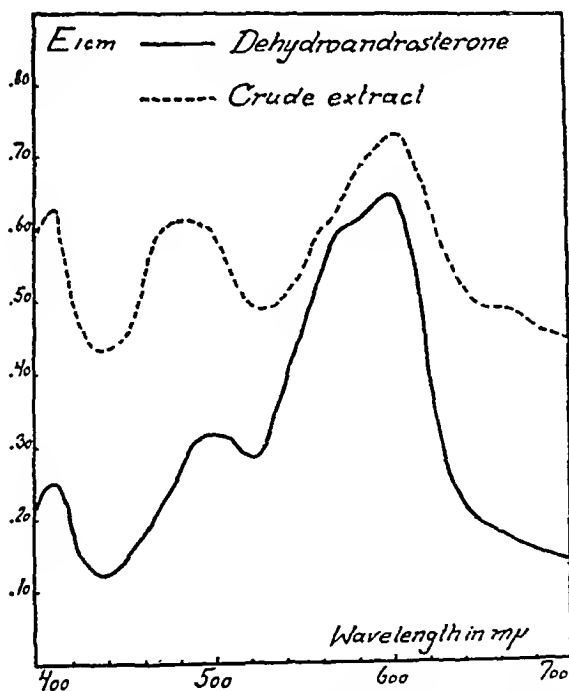


Fig. 1.

Absorption spectra for crude urine extract (containing 0.2 mg. total 17-ketosteroid) and for 0.2 mg. dehydroandrosterone after performing *Dirscherl & Zilliken's* reaction.

Abscissa: Wave length in m μ . Ordinate: Extinction coefficient.

Calculating on the basis of this reading we find that the crude extract contains 1120 mg. dehydroandrosterone. This is obviously too high, as the extract contained only 1000 mg. 17-ketosteroid. For the purpose of obtaining an approximate of how much the result was too high we made the following tests:

1. *Fractionating with digitonin.*

We treated 5 ml. of crude extract with digitonin by a micro-method described by *Frame* (1944). The resulting α - and β -fractions were subjected to the usual 17-ketosteroid reaction with m-dinitrobenzene. By this means the crude extract was found to contain a total of 320 mg. α -ketosteroid and 680 mg. β -ketosteroid. As all the dehydroandrosterone is contained in the β -fraction, the highest possible quantity in the extract must therefore be 680 mg.

2. *Antimony trichloride reaction according to Pincus (1943).*

We set up a reaction with the following substances: 0.01 ml. crude extract (corresponding to 0.1 mg 17-ketosteroid), 0.1 mg. androsterone and 0.1 mg. dehydroandrosterone. The reaction colours were examined photometrically as described above. The absorption curves are shown in fig. 2.

As *Pincus* states that this reaction is specific to androsterone and its isomers (isoandrosterone and aetiocholanolone), it is possible from these readings to calculate that the extract at most can contain 420 mg. dehydroandrosterone. However, a closer consideration of the absorption spectrum for the crude extract shows that the latter is not fully identical with that of any of the pure substances assayed. The significance of this will be gone into later.

For the present we must say that *Dirscherl & Zilliken's* reaction, at any rate directly, cannot be applied in the form as used here. The results arrived at are demonstrably too high. As is usually the case in such reactions, it would be natural to refer the excessive result to the formation of unspecific colour components resulting from impurities in the extract.

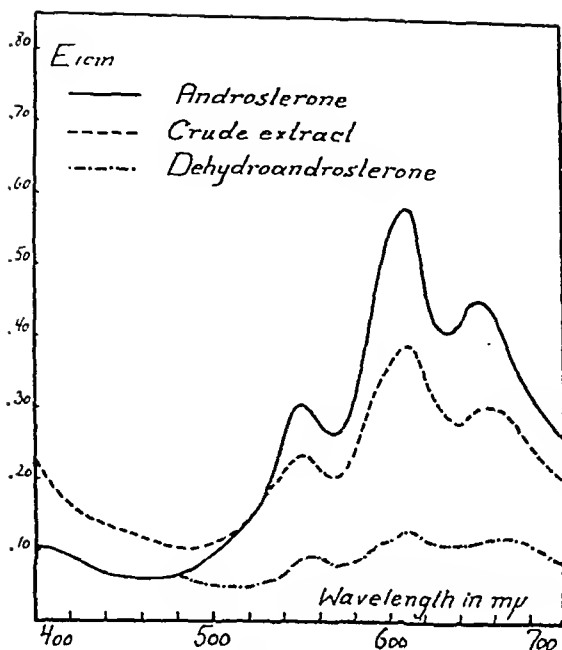


Fig. 2.

Absorption spectra for *Pincus'* SbCl_3 reaction performed on urine extract corresponding to 0.1 mg. 17-ketosteroid, and for the same amount of pure androsterone and dehydroandrosterone. Abscissa: Wave length in $\text{m}\mu$. Ordinate: Extinction coefficient.

We have tried various methods to avoid this. In the first place, it was reasonable to perform the reaction not on the crude extract but on the β -fraction obtained by the digitonin precipitation. Fig. 3 shows the result of a test of this kind, and for purposes of comparison we give absorption spectra for the α -fraction and the pure dehydroandrosterone.

The first to be observed from this experiment is that the β -fraction alone gives an absorption spectrum with a maximum at the same point as pure dehydroandrosterone. On the other hand, the curve for the α -fraction is very similar to the one obtained with the two isomer androsterones (*Theil Nielsen*, 1948). Secondly, the experiment shows a much lower content of dehydroandrosterone in our crude extract, about 650 mg.. This agrees very well with what was found by the 17-keto-

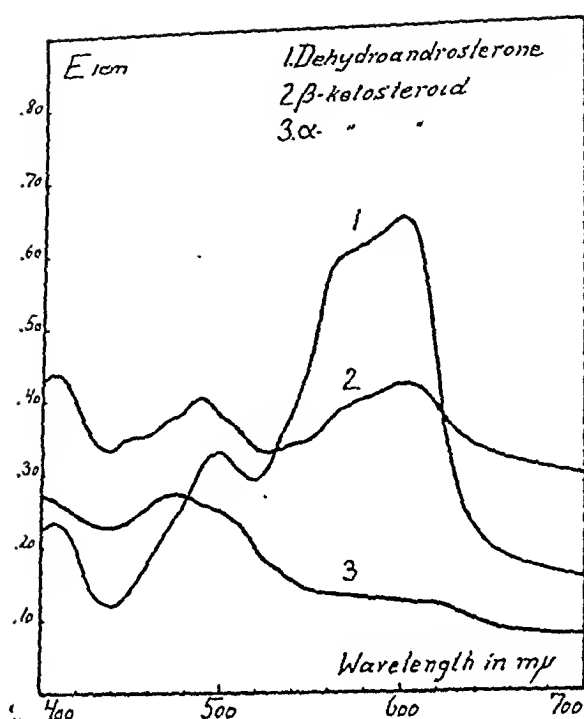


Fig. 3.

Absorption spectra (Dirscherl & Zilliken's reaction) for the following substances: 1) Dehydroandrosterone 0.2 mg.; 2) β fraction corresponding to 0.02 mg. crude extract; 3) α fraction corresponding to 0.02 mg. crude extract.

Abcissa: Wave length in $m\mu$. Ordinate: Extinction coefficient.

steroid determination on the β -fraction (680 mg.), but not so well with the *Pincus* reaction (420 mg.).

Of other purification possibilities we turned our attention particularly to chromatographic adsorption, a method often employed with good results in assaying 17-ketosteroids and similar substances. Recently *Dingemanse et al.* (1946) have tried applying the method in a modification intended to permit of the determining of the various 17-ketosteroids or groups of them. Having regard to certain remarks to be made in the discussion, we shall first make mention of an experiment carried out exactly in accordance with the directions of *Dingemanse et al.*: We evaporated 10 ml. crude extract to dryness. The residue was dissolved in 50 ml. benzene (stored over

sodium wire). The solution was poured on to a column of Al_2O_3 (Brockmann) having dimensions 15×100 mm.. Then 50 ml. portions of the following solvents were poured on in the order shown:

- 7 \times 50 ml. benzene
- 24 \times 50 ml. benzene with 0.1 per cent ethanol added
- 12 \times 50 ml. benzene with 0.5 per cent ethanol added
- 2 \times 50 ml. ethanol

Each fraction to be collected separately.

The 17-ketosteroid was determined on all the fractions by the m-dinitrobenzene reaction and the dehydroandrosterone by the sulphuric acid reaction in our modification.

In the ketosteroid assay the fractions Nos. 13—33 and Nos. 35—45 gave weak, brownish-yellow reaction colours and therefore are omitted from our calculations. In the sulphuric acid reaction for dehydroandrosterone the fractions Nos. 4—11 gave colours very similar to that obtained with pure dehydroandrosterone. The other fractions either gave no colour at all or a very pale yellow like the colour observed with the isomer androsterones; nevertheless, we would not venture to place them to that group. Fig. 4 shows absorption spectra for a selection of eluate fractions.

It is opportune here to draw attention to the fact that before setting up the sulphuric acid reaction on benzene eluates it is vital that all benzene is distilled off; otherwise we have found that the reaction either fails completely or is too weak.

We have visualized the results in a graph similar to that employed by *Dingemanse et al.*. In a coordinate system the eluate fractions are plotted in succession (Nos. 1—45) along the abscissa, and their content of total 17-ketosteroids and of dehydroandrosterone as the ordinate (fig. 5).

From this we can now calculate the content in the total crude extract (100 ml.) at:

17-ketosteroids (total)	981 mg
Dehydroandrosterone	700 mg

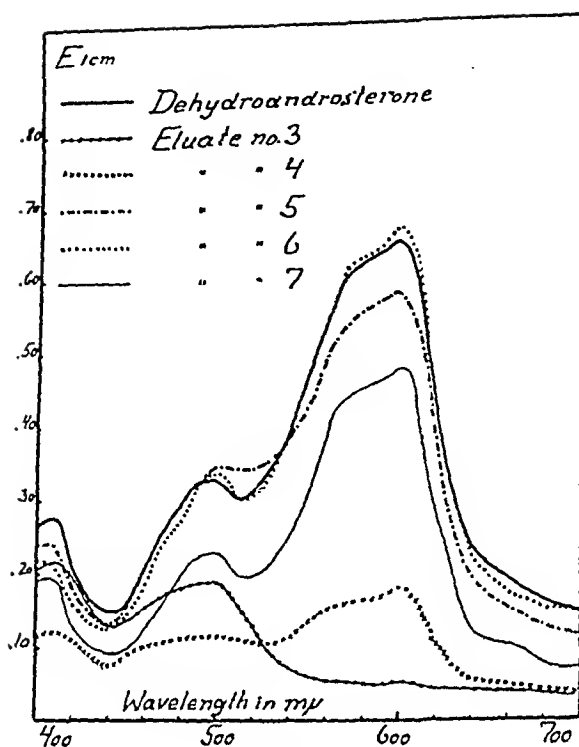


Fig. 4.

Absorption spectra plotted from dehydroandrosterone determinations by the sulphuric acid reaction on the eluate fractions Nos. 3—7.

0.5 ml. of each fraction is used.

Abscissa: Wave length in $m\mu$. Ordinate: Extinction coefficient.

The total 17-ketosteroid assay does not differ more from the original direct assay on the crude extract than is permitted by experimental error.

We have made experiments in which the chromatography itself was varied in different ways:

1. Elution with benzene-ethanol (1 %) and dividing the fractions according to colour (yellowish-reddish-colourless). Nearly all the dehydroandrosterone was found in the reddish fraction. In 100 ml. crude extract we found a total of 660 mg. dehydroandrosterone.

2. Elution with ether: The column is washed out more rapidly than when using benzene-ethanol mixtures, but the

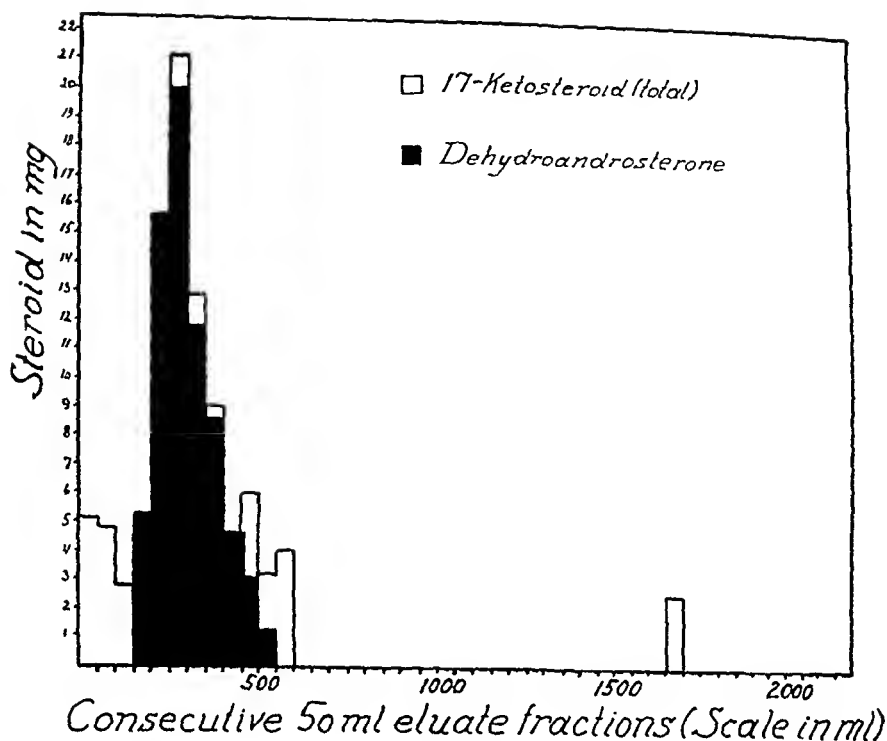


Fig. 5.

17-ketosteroid and dehydroandrosterone patterns obtained by chromatographic-spectrophotometric analysis of 10 ml. crude urine extract.

Abscissa: eluate fractions in ml. Ordinate: mg substance (17-ketosteroid-dehydroandrosterone in mg. in 50 ml. eluate fraction).

reaction colours obtained are not so close to that of pure dehydroandrosterone as those found in experiment 1. Nevertheless, the results are approximately the same: 715 mg. dehydroandrosterone in 100 ml. crude extract.

3. Elution with carbon tetrachloride-ethanol mixtures (see *Callow (1939)*) gives an equally good result.

DISCUSSION

Dirscherl & Zilliken's sulphuric acid reaction for dehydroandrosterone as modified by one of us (Aa. T. N.) was not

found to be directly applicable to the crude urine extract. After suitable purification by means of chromatography, however, we obtained reaction colours very similar to those obtained with pure dehydroandrosterone. The dehydroandrosterone content in the total crude extract calculated from this (700 mg.) agrees fairly well with the content of β -17-ketosteroids determined according to *Frame* (680 mg.), whereas there is less good agreement with the result arrived at by the *Pincus* method (420 mg.). In this connection it should be remarked in the first place that the *Pincus* reaction originally was devised only for determining isomer androsterones and not dehydroandrosterone. Therefore the latter must be determined indirectly, which may involve error in the assay. In the second place, as already stated the reaction colour obtained with the extract is not quite identical with that of the pure substances.

It might perhaps have been thought that the result by our method indicated the total β -17-ketosteroid content, i. e. that the isoandrosterone was also determined, whereas this was not so with the result obtained by applying the *Pincus* reaction. However, *Theil Nielsen* has shown that with the sulphuric acid reaction isoandrosterone does not give the colour characteristics of dehydroandrosterone.

On the basis of a graph like that shown in fig. 5 *Dingemanse et al.* claim to be able to calculate the content of individual 17-ketosteroids, though they consider there is an exception in the case of dehydroandrosterone and isoandrosterone, which cannot be separated by chromatography. Nevertheless, even with this reservation we are unable wholly to share their opinion. Apart from the dehydroandrosterone fraction, which is apparently fairly pure, we consider that the other fractions are too little well-marked and well-separated for conclusions to be drawn as to the identity of the fractions from a comparison of their curves.

It should be added that we have not found the fraction which *Dingemanse et al.* designate as Compound II. In this connection it must be emphasized, however, that our case is

a *non-malignant* tumour (adenoma), whereas the major part of cases described in the literature (including that of *Dingemanse et al.*) are true malignant tumours.

SUMMARY.

Urine from a woman with a virilizing benign adrenal cortical tumour (adenoma) is assayed spectrophotometrically.

The 24-hour 17-ketosteroid excretion, measured by the m-dinitrobenzene reaction, is 100 mg., about 70 % of which is β -17-ketosteroid.

By means of a modification of *Dirscherl & Zilliken's* colour reaction for dehydroandrosterone in conjunction with chromatographic adsorption it is demonstrated that the β -fraction consists of dehydroandrosterone.

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A CASE OF ADRENAL VIRILISM PERSISTING UNCHANGED AFTER EXCISION OF BILATERAL ADRENOCORTICAL ADENOMA

BY

A. LETH PEDERSEN

Adrenal virilism can be defined as the group within the adrenogenital syndrome where the effect of the adrenocortical hyperfunction is mainly one of virilisation affecting women after puberty.

Numerous single cases of the adrenogenital syndrome have been reported in the course of time, and also many excellent synopses dealing with this syndrome have been published, in particular within recent years. A detailed account of it will, therefore, be omitted here, but reference may be made to text-books of endocrinology (see a. o. *Goldzieher*, 1939, *Hoffman*, 1945 and *Nielsen*, 1938—42) or some of the synopses mentioned above (*Broster, Allen, Vines, Patterson, Greenwood, Marrian & Butler*, 1938, *Cahill*, 1944, *Cameron* 1947, *Dahl-Iversen & Hojensgaard*, 1947 and *Kenyon*, 1944).

However, the present case of adrenal virilism seems worth publishing because of certain facts of particular interest observed in this patient.

1. An enormously high androgen and 17-ketosteroid excretion.
2. The favourable response of the facial hypertrichosis to

treatment with large intramuscular injections of oestradiol monobenzoate.

3. The unchanged persistence of the syndrome after excision of adenomas in both adrenals.

CASE HISTORY

Neurological Dept., *Frederiksberg Hospital*. Case 130/46.

Divorced female worker in a box factory; born 1901.

Past history: No known cases of endocrine disturbances in the family. A sister has had psychogenic psychosis twice, but otherwise no cases of nervous or mental diseases in the family.

The patient had rheumatic fever ab. the age of 10 years with attending cardiac complication, but has had no cardiac symptoms since, and has always been of good health except for her present complaint.

The patient has from a child been rather hairy on her arms. The growth of hair was otherwise normal until the age of 20, when hairs began to grow on her upper lip and chin, and subsequently also on her cheeks. Some years later an abnormal growth of hair began to appear also on the breast, the remaining part of the body, and the extremities. The hypertrichosis increased steadily for 7 to 8 years and has since remained unchanged. About the age of 23 the patient began to grow bald. Ostensibly the loss of hair started after an accident when her hair had caught fire and some of it had been burnt away. The burning was inconsiderable, but after that she developed baldness of a typically male character, so that since the age of 29 she has had only a fringe of hair at the back of her head. If she did not remove the growth of hair on her face she would grow a beard like a man. She has applied epilation almost daily.

Almost simultaneously with the development of hypertrichosis the clitoris began to increase in size. It grew considerably in the course of a few years. The enlarged clitoris caused her no trouble during coitus, but affected her mentally. On the advice of her doctor she was, therefore, in April 1936,



Photo 1.
Before treatment.
(The pt. wears a wig — different in the two pictures).



Photo 2.
After *Ovar* treatment.

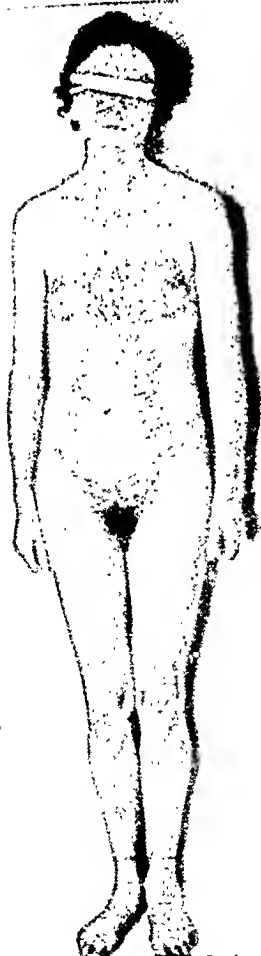
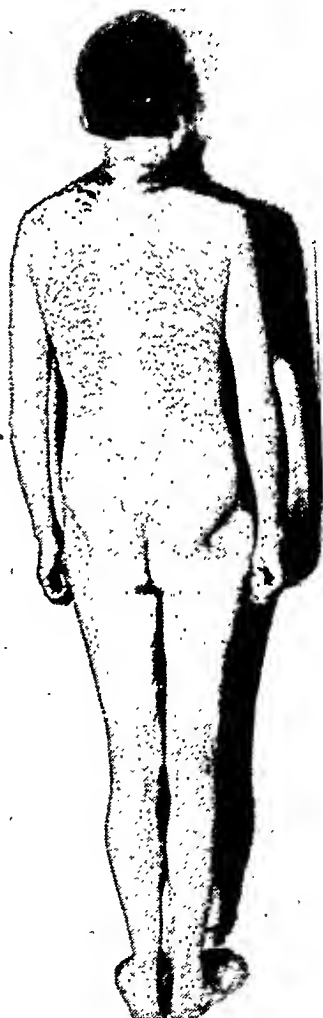


Photo 3 and 4.
Before treatment,
but the same after
treatment.



admitted to a surgical department, where the clitoris, 3 to 4 cm. long and ab. 1 cm. in transverse diameter, was extirpated. Her voice became deeper and coarser about the age of 20, but otherwise she has remarked no bodily changes.

Menses did not begin till about the age of 18. They have always been irregular, of 1 to 2 weeks' duration with intervals of from one to several months. She has never been pregnant. After 1935 increasing metrorrhagia, on account of which amputation of the supravaginal part of the uterus in October 1936. The body of the uterus was orange-sized and fibromatous. Ovaries and internal genitals were perfectly normal. A few years after the amputation of the uterus she began to suffer from hot flushes, which occurred continually through 5 or 6 years.

The patient's libido was always weak. She had not had sexual intercourse, nor been interested in erotic or sexual relations till about the age of 22, when she made her husband's acquaintance. Was married from 1924 to 1928, but then divorced her husband, because he played her false. While married she had sexual intercourse with her husband, comparatively rarely, indeed, but she believed to have obtained a *normal pleasure from it*. After the divorce the patient led a continent life until 1937, when she met a man, with whom she lived for 2 years. Within this period she had sexual intercourse at intervals from weeks to months. She felt some pleasure at it, at least no resentment against it. Since then she has had sexual intercourse only a few times without actually obtaining any pleasure from it. She no longer feels a sexual desire. Except for this gradual loss of libido, which was always weak, she has undergone no changes in a sexual respect, more particularly she has never felt homosexual or other morbid inclinations.

Mentally the patient was originally quiet, even-tempered, and cheerful, but after the divorce in 1928 she became of a more melancholy disposition, and would often be in low spirits. Since about 1930 she has been suffering from an almost constant frontal headache, and since 1940 she has developed

an ever increasing feeling of tiredness, and has, moreover, been suffering from giddiness, of the kind one may feel on a deck. No change has occurred in her personality or her psyche, apart from that elicited by the depression.

Because of these symptoms the patient was in January 1945 admitted to the Neurological Dept., *Frederiksberg Hospital*, to which she was admitted 4 times in all until February 1946.

Physical examination (on admission):

Mentally: very emotional and sensitive with a vein of depression.

Her voice is rather deep, somewhat rough and hoarse. Her features are somewhat coarse and clumsy. There is pronounced acne. On the whole her complexion bears the stamp of the daily epilation (see photo 1). There is marked blepharoconjunctivitis. The skeletal structure and the distribution of fatty tissue are of the male type, and the mammae are highly atrophic (see photos 3 and 4). Height 169 cm., weight 60 kg.

Growth of hair: On the head there is found only a 3 to 4 cm. broad fringe of hair from the back of the head to both ears. The rest of the head is completely bald, without cicatricial changes in the skin (the patient wears a wig). The eyebrows are rather thick, growing together over the nose. Eyelashes normal. The growth of hair on the body is very thick and »shaggy«, particularly so on breast and back, where the individual hairs reach a length of 5 and 3 cm. respectively (see photo 5). Axillary hairs normal. Pubes rather thick, but no particular hairiness along the linea alba, whereas the pubes are continuous with the growth of hair on abdomen and femora. The growth of hair round anus and genitals are of the male type. The growth of hair on the lower arms is very thick, and the hairs are up to 2 cm. long. The upper arms are less hairy. The femora likewise present a thick and up to 2 cm. long growth of hair, whereas that on the lower legs is less pronounced.

The medical and neurological examinations revealed nothing abnormal.



Photo 5.

Before treatment (but unchanged after treatment).

The eyes were likewise found to be normal, except for the blepharoconjunctivitis.

Ear and throat examination: The thyroid cartilage is strongly developed, of the male type, the angle between the laminae of the cartilage being 90° (the normal for women is 120°); otherwise nothing abnormal.

Gynaecological examination: Cicatrice after extirpation of clitoris, vagina normal, adnexa not palpable, movable uterus rest.

X-ray of skull: Pronounced digital impressions; no hyperostoses; sella normal. X-ray of kidneys and adrenals revealed normal conditions, normal pyelograms. X-ray of column: Light spondylosis deformans in the thoracolumbar part; otherwise nothing abnormal.

Electrocardiogram: Nothing definitely abnormal.

Glucose tolerance test (3 times): Fasting blood sugar normal, but the blood sugar level has not become quite normal after 3 hours; twice there was seen a rather considerable rise (maximally 202 and 182 mg. % respectively).

Basal metabolic rate: 134 to 122 %.

Blood pressure slightly increased, fluctuating between 175/110 and 150/80.

Serum protein determination (twice) showed normal values for total protein and albumin, while the globulin fraction was on the lower side of the normal.

Fractional cholesterol determination in serum (determined 3 times) revealed no definitely morbid conditions. Twice, however, there were found decidedly low values for total cholesterol.

Calcium, phosphorus, potassium, and sodium concentrations in serum normal. Serum phosphatase normal.

White and red blood corpuscles, index, differential counting, osmotic resistance, and capillary resistance normal.

At times the urine contained traces of albumin. Microscopy: Normal conditions. Blood urea and urea clearance normal.

Cerebrospinal fluid normal.

Hormone analysis on 24-hour urine: Excretion of gonadotrophine low (less than 5 R. U.). Oestrogen excretion normal (33 M. U.). Androgen excretion 602 C. U., and 17-ketosteroid excretion 286 mg. within 24 hours, in other words enormously increased (see later).

Summary of case history:

The patient is a woman, aged 44, who had been of normal health until the development of her present complaint about the age of 20, with almost universal hypertrichosis, masculine baldness, clitoris hypertrophy (extirpated 1936), irregular, but rather profuse menses (until amputation of the uterus 1936), a deeper voice, somewhat masculinized bodily type, and possibly decreasing libido. Except for this virilisation the patient presented no signs or symptoms of any kind. The signs of virilisation had become fully developed after 7 or 8 years and had persisted unchanged for 16 or 17 years, when I first saw the patient in 1945. Examination of the patient revealed, in addition to the virilisation, an enormously in-

creased androgen and 17-ketosteroid excretion, as well as a slightly increased basal metabolic rate, inconsiderable hypertension, and a somewhat lowered carbohydrate tolerance, but otherwise no unquestionably pathological features, neither on clinical nor on laboratory examinations.

On the basis of the above findings we judged the patient's affection to have been caused by a hyperfunction of the adrenal cortex. This hyperfunction seemed to be due rather to hyperplasia than to a tumour, chiefly on account of the protracted course and of the fact that no tumour could be demonstrated.

Treatment and Course:

Having previously tried to treat hypertrichosis in women with oestrogens, though, indeed with a somewhat uncertain result, I started with submitting this patient to such treatment, because I regarded her as particularly fit for it on account of her large androgen production. I treated her exclusively by oestrogen (Ovex) injections of 50,000 I. B. U. each (i. e. 5 mg. oestradiol monobenzoate dissolved in oil).*)

A total of 82 injections were given from April 9 to Oct. 16, 1945, at first 3 times weekly, and in the end once daily. The acne in the face had disappeared and the blepharitis been cured after 4 or 5 weeks (i. e. after 12 to 15 injections); but no effect was observed yet on the growth of hair. After well over 2 months, when ab. 30 injections had been given, the hypertrichosis began to respond to treatment, particularly so on face and neck, where the hairs grew lighter, scarcer, and easy to epilate. Also the hairs of the body seemed to sit more loosely. By the end of the course of treatment the effect on the hypertrichosis was evident on face and neck (see photo 2). Hairs did grow out here, indeed, but they were far smaller in number, much lighter, and much easier to epilate. While she had previously spent from 1 to 1½ hours daily with epilation she could now do with less than 5 minutes. The growth of

*) The *Ovex* applied was most kindly placed at my disposal by *Lovens kemiske Fabrik*, to which I bring my sincerest thanks.

hair on body and extremities had not decreased to any visible extent, but the individual hairs seemed to be looser.

The hypertrichosis on the face increased again after a pause of a few months in the *Ovar* treatment; but it never reached the former thickness. We now attempted X-ray on adrenals and pituitary body, prompted to this treatment among others by certain statements in the literature. A total of 1000 r distributed over 10 treatments were given first to each adrenal. Ab. 3 months later X-rays were applied to the pituitary body, 3 fields with a total of 300 r to each. No effect was observed, however, neither on the hypertrichosis nor on the other signs of the patient's virilisation.

The *Ovar* treatment was, therefore, resumed. Daily *Ovar* injections, a total of 24, were given from Jan. 28 to Febr. 21, 1946, after which the hypertrichosis on face and neck decreased, just like after the former *Ovar* treatment. After one month's pause in the treatment the hypertrichosis increased somewhat again. Hence *Ovar* was given from April 4 to July 1, 1946, about twice weekly, a total of 25 injections, and with the same favourable result as before.

After discontinuation of the treatment the growth of hair became a little thicker again. Since the patient suffered mentally under her affection, which must be supposed to be localized in the adrenals, either as hyperplasia or as a tumour, operation was decided on. In September 1946 the patient was, therefore, admitted to the Surgical Dept. C of the *State Hospital* (Prof. *Dahl-Iversen*). On Sept. 13, 1946 she was operated on in the left side. The left adrenal proved to have been transformed into a $15 \times 11 \times 8$ cm. large tumour, in which there was found no normal adrenal tissue, except for a narrow strip at the anterior lower pole. The entire adrenal was excised. Well over 2 months after the operation the growth of hair on face and neck had become thinner again, perhaps a little more so than after the previous *Ovar* treatments, whereas the hypertrichosis on body and extremities was unchanged. After 4 or 5 months, however, the patient presented increasing hypertrichosis on neck and face. As she was still excreting large

amounts of androgens and 17-ketosteroids she was operated on in the right side on March 3, 1947. A fist-sized capsulated adrenal tumour was found, which peeled readily from the adrenal. The tumour, measuring $9 \times 9 \times 5$ cm., was excised. Microscopy of this tumour, as well as of that removed at the former operation, revealed a typical adrenocortical adenoma with no signs of malignancy. Both operations ran an uncomplicated course with no suggestion of adrenal insufficiency (*Dahl-Iversen & Hojensgaard, 1947*).

A few months after the latter operation a decrease was observed in the hypertrichosis on face and neck, corresponding almost to that seen after a thorough *Ovar* treatment. On body and extremities, on the other hand, the hypertrichosis persisted unchanged. However, after yet another month the hypertrichosis on neck and face was seen to have increased somewhat again. The *Ovar* treatment was, therefore, resumed with injections twice weekly, a total of 23, from June 13 to Sept. 11, 1947. The treatment had an excellent effect on the facial hypertrichosis, even somewhat better than previous treatments, but none on that of body and extremities.

The growth of hair on face and neck is now (Jan. 1948) very thin, the hairs are light and very loose. The patient manages with epilation once weekly, and still there are but few hairs. Her complexion is clear (almost as photo No. 2). The baldness is unchanged, and the remaining hypertrichosis as when I first saw the patient. Mammæ, distribution of fatty tissue, and general habit are likewise unchanged.

The patient is in a depressed state of mind, very disappointed with the poor result of the operation, which she had hoped would have a great effect. She is very sensitive with inferiority complexes, very self-centred, and is always tormented by the idea that she is abnormal. Otherwise no mental changes. She feels as a woman, also sexually, but her libido has actually disappeared.

Table 1 summarizes the determinations of the output of 17-ketosteroids and of androgens.

The previously slightly increased blood pressure is now

Table 4.

	1945				1946		1947						
	Jan.	July	Sept.	Oct.	Sept. 13.	Oct.	Jan.	March 6.	March 8.	March 15.	May	Sept.	Nov.
17-ketost. in mg/24 h.	252	286	395	394	1. operation	80	202	2. operation	136	82	74	100*	212
androgen in C. U./24 h.		602	730			150	200		120	73	172		180

Excretion of 17-ketosteroids and androgens in the urine.)*

The excretion of 17-ketosteroids, indicated in milligrams, was determined photometrically, except that marked *, which was prepared in a chemically pure form.

The excretion of androgens was determined biologically on capons and indicated in capon's comb units (C. U.).

The figures in Table 1 indicate the average 24-hour excretion for 3 to 5 days. The 24-hour excretion was, however, often higher, once (July 8, 1945) even 582 mg. and 1360 C. U. respectively.

normal (about 120/80). There is no postural hypotension. The basal metabolic rate is likewise normal (107 %), and the glucose tolerance no longer definitely abnormal. These functions seem to have become normal after X-ray treatment of the pituitary body.

DISCUSSION

Androgen and 17-ketosteroid excretion.

The excretion of androgens as well as that of 17-keto-

*) The androgen excretion as well as that of 17-ketosteroids have most kindly been determined in *Lovens kemiske Fabrik*, for which I want to express my sincerest thanks to the factory and to M. Tonnesen, Dr. pharm., Director of the Biological Department. The androgens were determined biologically on capons, and the 17-ketosteroids photometrically according to *Callow's* method (see also *Jensen, C. C., Pedersen-Bjergaard, K. & Tonnesen, M.*: Bibliot. f. læger, separate issue Dec., 1944).

steroids, followed throughout the course, varied a great deal, but the variations were generally parallel for the two substances (see Table). The *Ovar* treatment had no definite influence on the androgen and 17-ketosteroid excretion, whereas each excision of an adrenocortical adenoma elicited a marked fall in both, followed, however, by a prompt rise, though not to the original amounts. The excreted 17-ketosteroids, both before and after excision of the adenomas, have been prepared in a pure form in *Lovens kemiske Fabrik*. The last time, after the excision (12th to 18th of Sept., 1947), the 24-hour excretion of 17-ketosteroids amounted to ab. 100mg. In both cases the substance consisted of 60 to 80 % dehydro-androsterone, while the rest was a mixture of different 17-ketosteroids (*Theil Nielsen, Pedersen-Bjergaard & Tonnesen*, 1948). In other words the β -fraction (consisting chiefly of dehydro-androsterone) is greatly increased in proportion to the remaining 17-ketosteroids, the former constituting normally no more than 10 % of the entire amount of 17-ketosteroids (*Salter, Cahen, Sappington & Sappington*, 1946). The findings in the present patient seem to accord well with previous statements (*Crooke & Callow*, 1939, *Friedgood*, 1944 and *Mason & Kepler*, 1945), according to which in particular the dehydro-androsterone concentration in the urine is said to be increased in association with adrenocortical tumours, unlike what is the case in hyperplasia. However, these statements seem to deal chiefly with malignant tumours versus hyperplasia. Other writers (*Salter, Cahen, Sappington & Sappington*, 1946) have found the β -fraction considerably increased, both absolutely and relatively, in proportion to the α -fraction also in association with adrenocortical hyperplasia, though less pronounced so than with adrenocortical tumours.

The androgen and 17-ketosteroid excretions found in this patient are enormously high. *Slot* (1936), in a woman with malignant adrenal tumour, found an excretion of 2200 I. U. per litre of urine (biologically determined), which corresponds to nearly 440 C. U. or 220 mg. *Slot's* finding is stated (*Cahill*, 1944 and *Goldzieher*, 1939) to be the highest reported so far.

Crooke & Callow (1939) found an excretion of up to 850 mg. 17-ketosteroid per litre of urine in a girl, aged 6, with left adrenocortical carcinoma; but the highest 24-hour excretion was 288 mg. *Westman* (1941), on the other hand, has published a case of adrenocortical adenoma, where no less than 20,000 C. U. were found per litre of urine.

Determinations of the androgen and 17-ketosteroid concentrations in *blood* are as yet rather uncertain, but various methods have been suggested and applied by a number of investigators (*Mc. Cullagh, Mc. Cullagh & Hicken*, 1931, *Mc. Cullagh & Osborn*, 1938, *Törnblom*, 1946 and *Zimmermann*, 1944). In *Lovens kemiske Fabrik* experiments have been made with the object of finding a method fit for the purpose, but no reliable method has been found so far. The enormous excretion of androgens presented by this patient induced us to think that she might also have a high androgen concentration in the blood and thus be found particularly fit for experiments of determination of androgens in the blood. The biological method (the capon's comb method) was applied for the determination. However, despite repeated experiments and although up to $\frac{1}{2}$ litre of blood was applied each time only very small amounts of androgens could be demonstrated, so small that they were demonstrable only by application on the capon's comb, but not by the injection method. The amount found did not exceed that present in the blood of adult men. This corresponds, however, to previous statements (*Crooke & Callow*, 1939 and *Törnblom*, 1946) of a normal serum 17-ketosteroid value despite large excretion in the urine, an observation which is suggestive of a low renal threshold value of these substances and a very prompt excretion. It likewise accords with the fact that the concentration of total cholesterol in the blood was not found increased in our patient.

Treatment. X-rays on adrenals and pituitary body have sometimes been applied with success in cases of the adrogenital syndrome (*Goldzieher*, 1939, p. 723). X-ray treatment of the adrenals proved to have no effect whatever in the case of

our patient. This is only what might be expected, since inhibition of the adrenal function must be supposed to be obtainable only by administration of such large X-ray doses that the surrounding organs are damaged. X-rays on the pituitary body, on the other hand, seemed to reduce the blood pressure, the basal metabolic rate, and the glucose tolerance to normal values, but had no influence on the virilism. This it not to be wondered at, since the latter was due to adrenocortical tumours. An effect might perhaps have been expected, if the syndrome had been caused by adrenocortical hyperplasia, which may possibly be due to an increased production of a corticotrophic hormone.

Also oestrogen in large doses has been stated sometimes to have a favourable effect on the adrenogenital syndrome and to reduce the 17-ketosteroid excretion (*Goldzieher*, 1939 and *Talbot et al.*, 1942). Our patient, however, presented no decrease of the androgen or the 17-ketosteroid excretion, despite prolonged treatment with large doses of oestrogen (*Ovex*). The *Ovex* treatment had a markedly inhibitory effect on the growth of hair in the patient's face, from which it could be kept away almost entirely, but no definite effect on the remaining hypertrichotic (virile) hairiness. The reason for this is unknown. It is, however, no doubt only a question of the quantity used, since a high dosage seemed to loosen the hairs on body and extremities, so it is not unlikely that an even higher dosage might have inhibited the growth of hair. The excretion, and therefore probably also the production, of androgens having remained unchanged during the *Ovex* treatment, the inhibitory effect of the latter on the facial hypertrichosis cannot have been due to a decrease in the androgen production, but must be supposed to have been caused by a direct antagonism between oestrogen and androgen (*Leth Pedersen*, 1947).

The oestrogen dose received by the patient was enormous. Within a scant 2½ years she was given 154 *Ovex* injections of 50,000 I. B. U. each (= 5 mg. oestradiol monobenzoate), or a total of 770 mg. After having got about 40 injections the patient developed oedema of feet and crura as well as petechiae in these areas, which, however, subsided shortly

after discontinuation of the treatment, not to recur despite renewed treatment with equally large doses as before. The treatment involved no other complications or troubles of any kind. If, however, the uterus had not been amputated, there would no doubt have been haemorrhage from this organ.

Diagnosis. It cannot be said for certain why excision of the two adrenocortical adenomas was not followed by recovery from the virilism, as we had expected. There is no clinical evidence to suggest that the syndrome should have been caused by a pituitary lesion (e. g. basophilic adenoma) in addition to the adrenocortical adenomas, nor that an ovarian tumour (arrhenoblastoma) should be present as well. The ovaries had also at previous laparotomy been seen to be normal. Moreover, the adrenogenital syndrome is never associated with such a large 17-ketosteroid excretion when caused by lesion in pituitary body or ovary.

Hence there can hardly be any doubt that the patient's virilism is still due to adrenocortical hyperfunction. It seems unlikely that the remaining right adrenal, which seemed normal after excision of the adenoma, should have become so hypertrophic that it can be supposed alone to produce such large amounts of 17-ketosteroids. Also metastases seem an unlikely possibility, since the removed tumours showed no signs of malignancy.

Therefore, I believe the most reasonable explanation to be one of hyperplasia or small tumours in accessory adrenals or aberrant adrenocortical tissue, of very common occurrence, most often situated close to the adrenals, along the spermatic veins, in ligamentum latum, ovaries, or testes. Cases of virilisation due to tumours in such accessory adrenals or aberrant adrenocortical tissue have previously been reported (Cahill, 1944 and Østergaard, 1946); but cases of concurrence of tumours in both normal adrenals and tumours or hyperplasia in accessory adrenals have to my knowledge not been described in the literature.

It seems doubtful whether the fact that the greater part

of the excreted 17-ketosteroids in our patient consisted of dehydro-androsterone also after excision of the adrenal adenomas can justify one in concluding that it is still tumour tissue which is responsible for the 17-ketosteroid production. The theory of the relative increase of the β -fraction in association with adrenocortical tumours, unlike what is the case in hyperplasia, seems applicable, if at all, only where it is a question of differential diagnosis between *malignant* adrenal tumours and hyperplasia. In the present case, as mentioned above, the tumours were hardly malignant. Therefore, if tumours are responsible, they must be of a benign nature (adenomas), and as such they should probably be regarded as forming part of a universal adrenocortical hyperplasia. The 17-ketosteroid excretion in our patient seems, therefore, to go against the above theory.

SUMMARY

A case is reported (woman, now aged 46) with a history of pronounced adrenal virilism of ab. 25 years' duration. She excreted enormous amounts of androgens and 17-ketosteroids in the urine, and the greater part of the 17-ketosteroids proved to be dehydro-androsterone.

X-rays on adrenals and pituitary body had no effect on the virilisation. By administration of large doses of oestradiol monobenzoate as intramuscular injections (*Ovaex*) it was possible to reduce the growth of hair on face and neck, while the treatment had no effect on the hypertrichosis on body and extremities. By operation first one adrenal was removed, which had been transformed into a large adenoma, and next a fairly large adenoma was excised from the other adrenal. But the virilism persisted unchanged, and the androgen and 17-ketosteroid excretion continued to be considerably increased.

The reason for the persisting virilism after excision of the adrenal adenomas is supposed to be the presence also of hyperplasia or tumours (adenomas) either in accessory adrenals or in aberrant adrenocortical tissue.

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ON THE MECHANISM OF THE ACTION OF OESTROGENIC HORMONE PREPARATIONS IN OVARIAN INSUFFICIENCY*)

BY

ERIK RYDBERG AND KAREN-MARGRETHE MATHIESEN

The very nature of the questions which are to be considered in the present paper makes us begin with something as fundamental as the properties by which we define a hormone.

A hormone may be regarded as a kind of activator — a substance which is produced in an organ and then when transported to some other organ or organs produces there an effect which is usually highly specific.

The very word »hormone« — meaning to excite or arouse — suggests that the action of such a substance in the organism is a stimulation.

But when speaking of sex hormone therapy one employs the word stimulation in another and special sense. As you will know, we often use the expressions »substitution therapy« and »stimulation therapy«, and regard these forms of treatment as entirely different in principle. Obviously, then, stimulation means something else and more than it does when we say that all hormonal activity really is a stimulation.

*) Paper read by E. R. at the meeting of the *Danish Society for Endocrinology* 29/4 1947.

When speaking of substitution- and stimulation therapy we are touching upon a question which is basic for the whole clinical sexual endocrinology and especially as regards the problems of which we are now to speak. It is therefore well to pause a moment and consider the fundamentals of the matter.

It is not so easy to give a concise definition that actually distinguishes between the concepts of stimulation therapy and substitution therapy. Let us try to express it as follows: We are dealing with *stimulation therapy* when by means of hormonal treatment we set in action a physiological mechanism which at the moment is at rest but otherwise functions spontaneously, and when this mechanism at least has a tendency to continue to function after the treatment. We may use the picture of a clockwork that is wound, but stopped. Giving the pendulum a push makes the clock go without the necessity of supplying additional force from without. This is stimulation treatment.

We speak of *substitution therapy*, however, when the hormone only acts directly on the receptor organ in a certain regular proportion to the administered amount of the hormonal substance and only for so long as it is being introduced into the organism or perhaps furnished from a depot if dealing with injection treatment.

Obviously it is desirable to use methods in the hormonal treatment which have the character of stimulation therapy if at all conditions are there to permit it.

Within the field of sex-endocrinological clinical research, investigators have from the beginning aimed, more or less consciously, at this object, but they have undoubtedly also in many instances believed that they had achieved an actual, more or less permanent stimulation of automatic organ functions when in reality they had only produced a *momentary* activity and the brief effects of this activation.

Actually, however, it is a very strong requirement that our therapy should be a true stimulation therapy, and within other fields of medicine than that of gynecology the endocrine therapy is nearly always pure substitution therapy and nothing

else. It is sufficient only to mention the insulin therapy in diabetes mellitus and the treatment with thyroïdin in myxedema and similar conditions; the fact is, that the actual function of the insufficient organs is not affected to any marked degree by these treatments, and the primary disease continues to exist.

It is undoubtedly the regular cyclical course in the female sexual functions that from the very beginning gave rise to a differentiation within the sexual endocrinology between substitution- and stimulation therapy. Disturbances of this functional course are evident, first of all, in that the menstruation ceases or becomes irregular, thus already in the first visible manifestation of this cycle, and the definite object of the treatment is therefore to help to re-establish the rhythmic function so that this function continues spontaneously.

Now, it is actually so that in many cases it is possible by hormonal treatment to achieve an effect corresponding to what is meant by stimulation therapy, e. g., when treating cases of ovarian insufficiency with gonadotrophic hormones (*Rydberg et al.* 1939, 1943 and *Westman*, 1941). In other cases it can be said beforehand that this treatment will not succeed, while a substitution therapy very well may be indicated and beneficial to the patient. It is a matter of pure substitution therapy, of course, when treating a climacteric patient for her menopausal symptoms or a castrate for the same well known syndrome. The state of function of the genital organs is not affected permanently, as there is no basis for an awakening of the cyclic processes. In the cases where there might be the possibility of achieving something more — i. e. in young women with the ovaries intact — it really becomes a question of cardinal importance where the hormone acts, and how far and deep its action extends.

We shall examine a little more closely the activity of the oestrogens in their clinical application from the point of view of stimulation- or substitution therapy in cases where a substitution therapy is conceivable and not a priori out of the question.

It is well known that the first experiments on the treatment of conditions of ovarian dysfunction involved the use of oestrogenic substances alone or in combination with corpus luteum hormone, so that the problem is by no means a new one.

Now, which effect could such a treatment be imagined to produce in a case of ovarian insufficiency, let us say in a typical case of secondary amenorrhea in a young woman?

We know that with sufficiently large doses it is possible to produce bleeding, and that the endometrium can be first brought into proliferation — by means of an oestrogenic hormone — and next into premenstrual change — by means of corpus luteum hormone — and this in castrated women. In castrates it is of course impossible to produce more than the peripheral reaction — a lasting stimulation and a spontaneously continuing cycle are out of the question because the ovaries are lacking. But what happens if we treat a woman who is suffering from secondary amenorrhea, and how shall we be able to gain insight into the mechanism of the mode of action of the oestrogens in cases where the ovaries are intact and where there therefore is a possibility of making them function?

This is really the crux of the matter as regards treatment of such cases, and the value of this kind of therapy depends on whether or not we have a chance of achieving more than the peripheral reaction. In a word, is the treatment of the ovarian insufficiency with ovarian hormone stimulation therapy or substitution therapy? On this point opinions have varied.

Kaufmann (1935, 1937) who has treated large series of amenorrheic patients with oestrogens and with oestrogens combined with corpus luteum hormone, is of the opinion that one must regard the treatment as more than pure and simple substitution therapy, but he does not attempt any further analysis of how these hormonal preparations act in the organism. *Westman*, who has reported his results in a comprehensive paper in 1941, shares the opinion of *Kaufmann* regarding the mode of action of these hormones. As early as in 1935

Zondek succeeded in producing a menstruation in a patient with primary amenorrhea by treating her with gonadotrophic hormone prepared from the urine of pregnant women, and he says on this occasion that he believes that the gonadotrophic hormones should make a true stimulation therapy possible in conditions of ovarian insufficiency, in contrast to what can be obtained by the then generally used treatment with ovarian hormones, which he regards as pure substitution therapy. We shall mention one other representative author who recently has given an excellent comprehensive presentation of the treatment with sex hormones and who has tried to appraise the present state of affairs as regards the therapeutic possibilities of these hormones. We are referring to the English investigator *Bishop*, who in 1944 says: »In the past ten years, however, there has developed a sober recognition of the fact that oestrogens are incapable of stimulating either the ovary or the pituitary...«

We shall refrain from a detailed critical examination of the basis for these statements. On the whole, *Kaufmann's* and *Westman's* results are in agreement, and what can be said in support of their claim — that the treatment with ovarian hormones in many cases is more than a mere substitution therapy — is the fact that in a relatively large number of cases of amenorrhea it has been possible not only to produce bleeding, which in itself is not so remarkable, but to start a cycle which then continues spontaneously.

When *Westman* (1941) has been able to produce not only one bleeding, but regularly recurring cyclic bleedings in 30 out of 64 cases of secondary amenorrhea, one must say that this result favors the idea that the treatment is not merely a momentary substitution for the reduced or totally absent ovarian function, but is something which in a more profound and more lasting way has affected the cyclic sexual functions.

An evaluation of the therapeutic results becomes difficult, however, because the majority of cases in which the ovarian function is set in action are within the group of patients who have suffered from an amenorrhea of relatively short dura-

tion. Moreover, when judging the results of the therapy one must take into account that the treatment, which consists of repeated series of injections, often will extend over so long a period of time that a spontaneous reappearance of the ovarian function can occur in several of these cases. But all in all, one must say, at any rate, that such works as that of *Westman* and that of *Kaufmann* make it highly probable that the treatment with ovarian hormones gives more than an accidental peripheral reaction.

We shall now present some cases which in our opinion support this point of view in a rather convincing manner.

One difficulty of judging the possible reaction of the ovary to the oestrogen treatment is that when using ordinary injection therapy with genuine ovarian hormone preparations dissolved in oil one cannot reckon with obtaining definite information from hormone analyses in connection with the treatment. First of all, this oestrogen must be given in the form of injections, otherwise it is broken down to the extent of 90—95 per cent in the liver and thus only to a very slight degree absorbed by the circulating blood, and when one introduces a larger depot of it dissolved in oil, the hormone is absorbed but slowly and will undoubtedly remain for a relatively long, indefinite period of time in the depot. If now the oestrogen excretion in the urine is determined soon after the treatment, one never knows whether the substance determined simply is the injected hormone or originates from the ovaries of the patient which have begun to give off oestrogen. The oestrogen found in the urine can, of course, originate from both of these sources.

Here the synthetic oestrogenic preparations have presented us with new possibilities for studying the reaction of the ovary. The fact is that these preparations — at least in so far as the one used by us is concerned — when given per os seems to pass the liver without being broken down, and moreover disappears from the organism within 24 or at the most 48 hours. The preparation to which we refer is »*Sexadien*« or di(p-oxyphényl)-hexadien. Its behavior in animals has been studied by Dr. K. *Pedersen-Bjergaard* (1940) and we have later found that

it behaves similarly in human beings. Table 1 shows some figures which indicate how rapidly this oestrogenic substance disappears from the urine.

Table 1.

Day	Sexadien administered orally	Twenty-four-hour output of oestrogens in mouse units	
		A	B
1	—	10	< 10
2	7.5 mg	800	200
3	7.5 mg	800	500
4	7.5 mg	350	250
5	—	300	600
6	—	< 10	10
7	—	< 10	10
8	—	< 10	10
9	—	< 10	< 10
10	—	< 10	< 10

Twenty-four-hour excretion of Sexadien in mouse units in two male persons (A and B). The determination of oestrogens (Sexadien) in the urine was begun the day before the treatment. The Sexadien was given perorally during 3 days. Dosage 7.5 mg. daily.

If substances with oestrogenic action are found in the urine, say 3—4—5 days after a Sexadien treatment has ceased, their origin cannot be the oestrogen administered but must be looked for somewhere else and the same is true even if large doses of Sexadien are used.

This means that we can conduct hormone analyses a few days after the treatment has been terminated, and that we, if we find a distinct increase in the excretion of oestrogenic substances after the treatment compared to that found before, can conclude that the increase is due to a hormonal source in the organism itself — in other words, that the ovary most likely has been stimulated to produce oestrogen.

We shall now show how the hormone excretion has behaved in some cases of amenorrhoea which we have treated with Sexadien. We have also examined the endometrium at the

bleedings that occurred in connection with the treatment, and we shall see that both methods of investigation give information that may serve in broadening our insight into what happens in the organism in these treatments.

OWN INVESTIGATIONS

In all we have treated 49 cases of amenorrhea with Sexadien.

The treatment has consisted of giving the Sexadien per os, 4—6 mg, in most instances 5 mg, daily. This treatment has been continued for 20 days or 3 weeks, so that the patients in each series have received about 100 mg. In several instances such series have been repeated several times, at intervals of a few weeks.

In 18 cases treated in this way we have undertaken determinations of the output of oestrogens before and after the treatment. 48-hours portions of the urine are used for the determinations and the values are given in mouse units per 24 hours — in one case (chart 5) only the average excretion during 10 days before and after the treatment are determined. The determinations of the urine hormonal content are performed at the biological laboratory of Løvens kemiske Fabrik, Copenhagen after the method of *Kemp & Pedersen-Bjergaard*, a short description of which is given in the paper by *Rydberg & Pedersen-Bjergaard* of 1943. In all cases but one the hormonal analyses after the treatment were made so late that the Sexadien given must have disappeared — in one case (chart 4) the first values of the determinations after the treatment possibly depending upon the Sexadien given during the days before.

The results of these hormonal analyses were as follows:

- I. Abnormally low values before the treatment
and unchanged low values after the treatment
in 8 cases
- II. Abnormally low values before the treatment

- and a slight but hardly significant rise of the output after the treatment in 3 cases
- III. Low values before the treatment and lower values after the treatment in 2 cases
- IV. Abnormally low values before the treatment and definite rise of the output after the treatment 5 cases

The values for the 2 cases of group III were the following:

1	{	Patient I. N. 24 hour output of oestrogens before the treatment	8—33 M. U.
		» I. N. 24 hour output of oestrogens after the treatment	8—11 M. U.
2	{	Patient R. H. 24 hour output of oestrogens before the treatment	8—67 M. U.
		» R. H. 24 hour output of oestrogens after the treatment	8—33 M. U.

In the groups I, II and III the values have thus been unchanged or fluctuating a little in the one or the other direction, when compared before and after the treatment. Such fluctuations are of course to be expected and cannot be claimed to lessen the significance of the curves which are reproduced in the charts 1—5.

In the following charts the output of oestrogens before the treatment and after can be seen. The stepped curve gives the oestrogen output in the urine per day in mouse units. When this curve is drawn as a dotted line it means that the actual values were not determined and that at any rate they fall below the value indicated by the dotted line.

The charts 1—5 show that there can be a considerable increase in the excretion of oestrogenic substances in the urine for a while after the Sexadien has been given in large doses to a patient suffering from ovarian insufficiency.

This increased excretion has been found so long after the treatment that it cannot be an expression for the excretion of the hormone ingested by the patient.

It might perhaps be argued that the metabolism — the

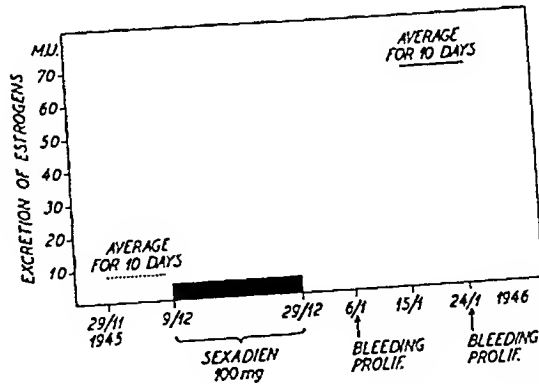
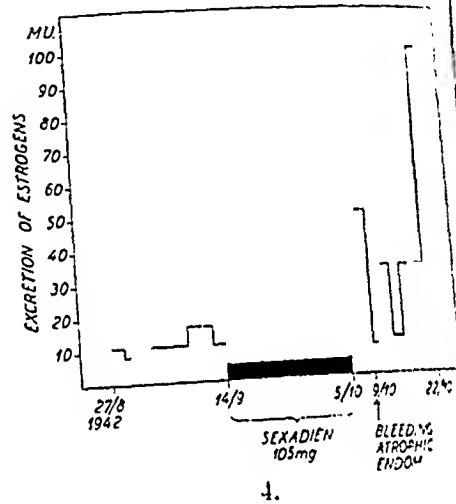
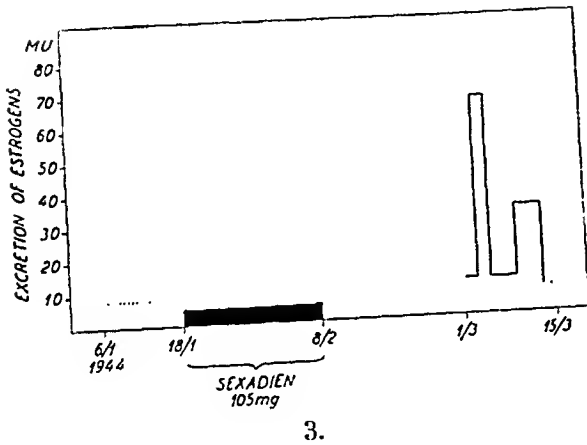
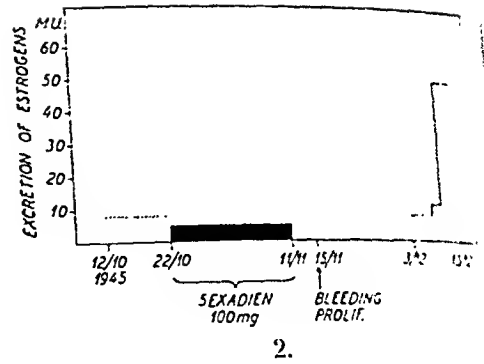
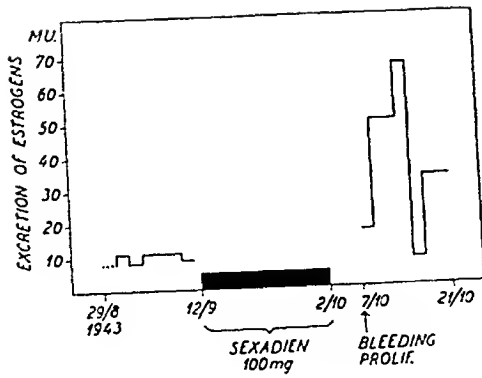


Fig. 1—5.

Charts, showing the excretion of oestrogens before and after the treatment in the 5 cases belonging to group IV.

degradation and excretion — of the oestrogen administered in some cases of amenorrhea is entirely different from what it is in men and in those cases of ovarian insufficiency where the hormone disappears from the organism and is not to be found in the urine a few days later. But that seems very far-fetched, and in our opinion there can be no doubt that this increased excretion of oestrogenic substances in the urine relatively long after the treatment must be due to an activation of the ovary. The experiments mentioned do not show how this is brought about, but one would naturally imagine that the activation probably occurs by way of the pituitary gland, i. e., that the administration of the oestrogen incites processes in the pituitary gland which in turn cause the stagnation to be broken. We have no analogies from animal experiments, so we shall refrain from suggesting any detailed theories pertaining to the mechanism involved in the activation of the pituitary gland.

We shall, however, refer to the work of *O. Watkins Smith* (1944, 1945) dealing with the pituitary response to oxidative inactivation products of the natural oestrogens which has a strong bearing upon the present questions.

If we now look at the reaction of the endometrium we find it to be very inconstant. Bleeding occurs with relative regularity after a Sexadien treatment like the one just mentioned, occasionally even while the treatment is in progress, but the anatomical picture of the endometrium is highly variable and is not in any definite relation to whether or not there occurs a rise of the excretion of oestrogens after the treatment. Very frequently there is a bleeding without the endometrium showing any sign whatever of having been affected by the hormone. In biopsy it occurred time and again that no mucous membrane was obtained, undoubtedly because of advanced atrophy. In other cases we obtained a few very small fragments of the endometrium which are just enough for the making of a microscopic preparation. When looked at, the slide shows a completely resting atrophic mucous membrane. One may also meet the anatomical picture of the proliferative phase but we

have rather seldom found a pronounced secretion picture in the endometrium of these cases.

We have re-examined the preparations from our therapeutic experiments with Sexadien, and when we include only the specimens obtained at bleeding in direct connection with the treatment, we find that the endometrium has exhibited the types recorded in the table 2.

Table 2.
49 Cases of Amenorrhea treated with »Sexadien«.

Number of biopsies	80
Atrophic or proliferative endometrium	70
Secretory changes	10*)
Pregnancies immediately following treatment	4

*) 4 specimens from the same patient.

Thus we find varying pictures of the endometrium, and in relatively few instances the picture of the mature premenstrual phase.

If one has observed a number of endometrial specimens obtained from patients suffering from ovarian insufficiency, whether or not in connection with hormonal treatment, then it seems to us that one must conclude that very often the bleeding has nothing at all to do with the phase development in the endometrium, and one is obliged, we feel, to assume that there must exist a — probably completely vascularly dependent — bleeding mechanism which very likely is under the influence of fluctuations in the supply of oestrogenic substances.

Relatively often the bleeding has occurred while the Sexadien administration was still in progress, but most commonly it follows a few days after the treatment has been discontinued.

As we have seen, in only 10 out of 80 specimens it was possible to observe a secretory phase in the endometrium. The closer scrutiny of the cases makes it seem probable that this change of the endometrium must be regarded as an effect of the treatment. Then one must imagine that there has been an activation of the pituitary gland and a giving off of luteinizing hormone which has acted on the ovary, since Sexadien alone cannot possibly have such an effect.

At present we dare not say anything definite regarding the therapeutic results. The biopsies show that the bleedings occurring in connection with the treatments rather seldom correspond to the menstrual change of the endometrium. This stands out in clear contrast to what is found after treatment with gonadotrophic hormones. The results at long sight do not appear to be very good either, but several patients have interrupted the treatment after one or two series and we have not performed a regular follow-up examination, our material being as yet too small.

We believe, however, that we are safe in saying so much: With Sexadien treatment like the one mentioned there is a chance to activate the ovary in cases of ovarian insufficiency, but the results of this treatment are by far not comparable to the results obtained with gonadotrophic hormones when given in rational dosages (*Rydberg et al.* 1939, 1943).

As seen from the table 2 we have 4 cases in which pregnancy occurred in close connection with the treatment. These cases most certainly suggest that the Sexadien treatment can bring about an actual activation of the ovary, an activation which also involves, or at any rate sooner or later may be followed by the phenomenon of ovulation. We shall therefore give some brief summaries of these 4 cases, and at the same time show a summary of a case where the pregnancy did not occur immediately after the treatment, but nevertheless, as we shall see, in all probability was due to the treatment.

Case records I—V.

Case I.

1161/42. R. A. Age 25 (Jan. 1944), married.

Menses began at 14, always irregular with interval from 3 to 12 months. Married for nearly two years. Involuntarily sterile.

Physical examination: normal findings.

1944 3/1—14/1	Oestr.: 11 50 <8 11 <8 11 M. U./24 hours.*)
	Gonad: <5 10 5 <5 <5 5 R. U./24 hours.
17/1—6/2	<i>Sexadien</i> 4 mg. daily, total dosage 84 mg.
10/2—17/2	<i>Bleeding.</i>

*) The figures are average for two 24-hours portions of the urine.

- 1/3—13/3 Oestr.: 67 100 67 67 33 100 M. U./24 hours.
Gonad.: 15 90 180 180 180 450 R. U./24 hours.
- 7/3 *Bleeding*. Endometrial biopsy. Insufficient quantity removed for histological diagnosis.
- 6/4 *Bleeding*. Continuing and increasing the following days.
- 12/4 *Abortion*. Evacuation.
Hereafter regular menstruation. Latest report 15/8 1946.

Case II.

883/46. I. O. Age 22, married.

Menses began at 13, always irregular with prolonged intervals. Latest spontaneous bleeding December 1945. In the beginning of April 1946 bleeding after »Ovex«-injections (oestradiol benz.). Never pregnant. Involuntarily sterile for 3 years.

Physical examination: normal findings.

- 1946 7/6 Endometrial biopsy: proliferation.
- 15/6—4/7 *Sexadien* 5 mg. daily, total dosage 100 mg.
- 9/6—11/6 *Bleeding*.
- 15/7—3/8 *Sexadien* 5 mg. daily, total dosage 100 mg.
- 13/8—1/9 *Sexadien* 5 mg. daily, total dosage 100 mg.
- 11/9 *Pregnancy* stated. Uterus of the size of an orange.

Case III.

1128/45. G. N. R. Age 28, married.

Two parturitions. Amenorrhoea after the latest parturition 30/6 1944. Treatment begun 14 months afterwards. Lactation during about 9 months.

Physical examination: normal findings.

- 1945 31/8 *Bleeding*. Endometrial biopsy: atrophic endometrium.
- 10/9—30/9 *Sexadien* 5 mg. daily, total dosage 105 mg.
In the first days of October scant bleeding.
- 24/11—14/12 *Sexadien* 5 mg. daily, total dosage 105 mg. No bleeding followed.
- 1946 28/1 *Pregnancy* stated.

Case IV.

3550/44. G. K. P. Age 29, married.

2 children, 3 abortions. The latest abortion Jan. 1944. Amenorrhoea during 1 year before treatment.

Physical examination: normal findings.

- 1945 5/1—25/1 *Sexadien* 5 mg. daily, total dosage 105 mg.
 18/1 *Bleeding*. Endometrial biopsy: proliferation.
 1/3—21/3 *Sexadien* 5 mg. daily, total dosage 105 mg. No bleeding followed.
Pregnancy stated.
 Parturition at term 3/2 1946.

Case V.

1828/44. I. H. Age 32, single.

Up to the latest two years menstruation regular. Then scant bleedings with an interval of 3—4 months. Amenorrhea 5 months before treatment.

Physical examination: uterus a little hypoplastic, otherwise normal findings.

- 1944 7/7—27/7 *Sexadien* 6 mg. daily, total dosage 126 mg.
 22/7 *Bleeding*. Endometrial biopsy: proliferation.

Hereafter regular bleedings of normal menstrual type for nearly 11½ year. Latest menstruation in the beginning of November 1945. *Then pregnant.*

That the pregnancies are associated with the treatment must be regarded as obvious, at least in so far as the first 2 cases are concerned, and in cases where ovulation thus has occurred after the treatment there can hardly be any doubt as to the activation of the pituitary gland. It is certainly not very probable that ovulation should be a direct oestrogenic effect on the ovary — at any rate, there is nothing in the experimental biology to support such an idea.

DISCUSSION

The following may be said in summarizing the hormone-analytical investigations and the closer observation of the cases in which pregnancy has occurred in connection with the treatment:

It is beyond doubt that oestrogenic acting hormones — here the synthetic preparation *Sexadien* — in certain cases of stagnant ovarian function can activate the ovary. This

activation can also be followed by ovulation. This means that with oestrogenic hormones we have a chance of performing a true stimulation therapy. Or one may express it as follows: The first provision for a stimulation therapy is that the hormone extends its activity beyond the peripheral genital organs, and that we thus can notice an effect on the ovary, the pituitary gland, or on both organs. The hormone-analytical investigations show that the ovary can be activated by Sexadien treatment, and we can say — *inter alia* because we have achieved several conceptions in direct connection with the treatment — that it is highly probable that the whole play is set in action by an activation of the pituitary gland.

Considering now the two types of hormonal treatment — with gonadotrophic hormones or with oestrogenic acting substances — then it can undoubtedly be said that we have been more successful with the gonadotrophins irrespective of the purely symptomatic treatment. On the other hand, however, our present experience strongly suggests that the essential difference as regards an effect on the internal secretory system is not so very great.

We may now roughly schematize the hormonal relations between the pituitary gland, the ovary and the peripheral organs as in figure 6.

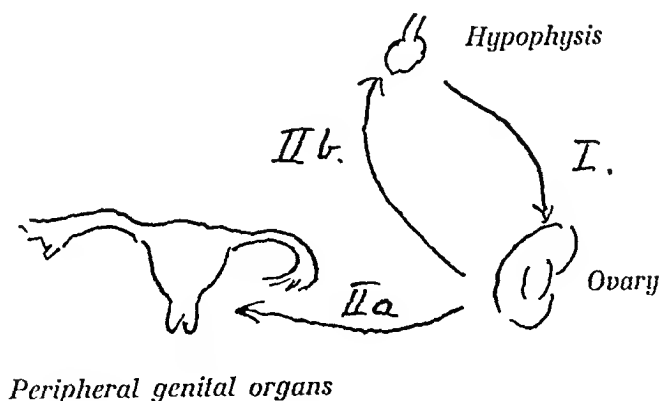


Fig. 6.

Hormonal Relations between the Pituitary Gland, the Ovary and the peripheral Organs.

When treating ovarian insufficiency hormonally we have, when setting in our treatment on track I in the figure, i. e., when using gonadotrophic hormones, a great chance of obtaining results which correspond to what we understand by stimulation therapy. When employing oestrogenic substances the hormone always acts upon the peripheral genital organs (track II a) but it is extremely probable that the treatment sometimes is set in on track II b too. At any rate, it can be said that when giving oestrogenic substances to a young and sexually mature woman suffering from temporary ovarian insufficiency her reaction sometimes will show that they do not *merely* act by the way of track II a, which might be called a »dead-end« track.

The closed reaction circuit I—II b suggests an apparatus which may be made to vibrate and which is capable of resonance. It should then be possible to produce resonance by impulses both along track I and along track II b.

To return to the picture of a clockwork, then it looks as if both the treatment with gonadotrophic hormones and with oestrogenic substances can function as the push which is required to set the clock going, and we may thus manipulate with the clockwork in different places and obtain the same result.

SUMMARY

The synthetic preparation Sexadien, di(p-oxyphenyl)-hexadien has been used in doses of 4—6 mg. daily in periods of about 3 weeks in 49 cases of amenorrhea.

Hormone-analytical investigations in 18 cases give strong evidence of a real activation of the ovary in 5 of these cases.

In 4 cases pregnancy occurred in close connection to the treatment.

These experiences are strong supports for the conception of the treatment with oestrogenic preparations being sometimes more than pure substitution therapy.

The chances for practical results in the treatment of ovarian

dysfunctional conditions in younger women are however apparently much better when gonadotrophins in rational dosage are given.

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URINARY EXCRETION OF OESTROGENIC SUBSTANCES AND 17-KETOSTEROIDS IN CASES OF METROPATHIA HAEMORRHAGICA*)

BY

MIRJAM FURUHJELM

Typical cases of metropathia haemorrhagica exhibit a hyperplastic endometrium containing cystic glands, and in case of bleeding thrombosed blood vessels and diffuse areas of necrosis. The ovaries in these cases may be slightly enlarged, and contain from few to many cysts representing various stages of follicular atresia, the most characteristic feature being an absence of corpora lutea. In all of these patients a failure of ovulation is present, and there is reason to believe, that the pathological changes in the endometrium are due to an exclusive estrogenic action. The reports published up to the present do not reveal neither whether this action is continuous or fluctuating, nor how strong it is. Therefore, it seemed to us of interest to investigate in patients suffering from metropathia haemorrhagica the urinary excretion of oestrogenic substances. If an exclusive increased production of oestrogenic substances is present in these cases, it may be expected, that the normal action of the pituitary body on other

*) This investigation has been carried out with the support of *Stiftelsen Therese och Johan Anderssons Minne*, Stockholm, for which I beg to offer my sincere thanks.

endocrine glands than the ovary is also influenced. These determinations were therefore completed in all cases with the determination of the excreted 17-ketosteroids.

In those cases where the endometrium is showing the very same pathological picture, there is a striking difference in the histological and gross appearance of the ovaries showing changes from cyst formations to atrophy. These facts are suggesting, that the ovary has a decisive importance in the pathophysiology of the disease.

The pathogenesis of metropathia haemorrhagica is unknown. Numerous explanations have been advanced. *Kurzrok* (1941) considered, that the disease may be caused by some endometrial disorder. *Meyer* (1920), *Schröder* (1919) and others believe, that it must be connected with irregular ovarian function. According to the theory advanced by *Westman* (1943) some disturbances in the production of gonadotrophic hormones may be responsible for the disease, probably due to an excited hypothalamico-hypophyseal function.

Only a few investigations have been done on the urinary excretion of oestrogenic substances in cases of metropathia haemorrhagica. Unfortunately most of these determinations have been carried out without hydrolysis of the urine. Therefore the values obtained were considerably too low.

In single determinations *Siebke* (1931), *Frank* (1931) and *Damm* (1936) observed increased levels. In a later study on 84 cases of metropathia haemorrhagica (*Genell*, 1941) confirmed their results, finding however higher levels in preclimacteric patients than in juvenile cases.

MATERIAL AND TECHNIQUE

The entire urinary output from 15 patients suffering from metropathia haemorrhagica was collected over a period of 20—30 days. Each determination was carried out on the total amount of urine excreted during 48 hrs. Particular details of the methods employed have been previously published (*Furuhjelm*, 1940). The amount of oestrogenic substances is

expressed in I. U. per daily output of urine. The determinations of the 17-ketosteroids were done with the method described by Zimmermann slightly modified by us (*Furuhjelm, 1940*). The amount of 17-ketosteroids are expressed in mg per daily output of urine.

All patients showed the typical picture of metropathia haemorrhagica with the endometrium of the classical type characterised by hyperproliferation and cystic glands. Two cases, however, showed signs of secretion in the glandular epithelium. The state of the endometrium was checked by biopsy and the determinations of oestrogenic substances and 17-ketosteroids were carried out during both bleeding and amenorrhea periods. The material is divided into three groups. Group 1 covers the patients treated only by curettage. The amount of the excreted substances in this group is presented in table 1 and in figure 1. Group 2 includes the patients subjected to omental implantation in the ovaries. The results are given in figure 2. Finally the results of patients treated with prominal are listed in figure 3.

RESULTS

Group 1. Patients treated by curettage only.

The urinary excretion of oestrogenic substances was considerably higher than in normal women, showing no decrease during bleeding periods. (fig. 1.)

In six cases the oestrogenic excretion was checked at intervals of up to one year, and it has been found, that the high rate of excretion remains unchanged. (table 1, fig. 2 & 3.)

Group 2. Patients treated with omental implantation in the ovaries.

Omental flaps were implanted in the ovaries in 5 cases (nos 4, 5, 7, 12 and 13) according to the method described by Westman (1946). The oestrogenic substances are in these cases carried by the blood stream via the portal system to the liver where they are partly inactivated. Using this method

Table 1.

Case no.	Age years	Duration of disease yrs	Urinary excretion in 24 hrs			
			Oestrogenic substances I. U.		17-ketosteroids mg	
				Mean		Mean
1	13	1/2	140, 140, 160, 100, 120, 125, 110, 150, 75, 40, 90, 120, 125, after 1/2 year 90, 50, 16, 130, 82	104	6.0, 8.0, 6.5, 6.0, 6.5, 6.0, 6.0, 5.0, 3.0, 2.5, 5.0, 5.0, 7.5, after 1/2 year 4.7, 2.2, 1.7, 2.4, 5.2	5.0
2	15	1/2	65, 65,	65	5.0, 3.5	4.3
3	18	2	70	70	1.8	1.8
4	18	4	100, 100, 120,	107		
5	18		90, 100, 90, 125, 180, 180, 180, 190, after 3 months 110, 120, 75, 120, 200, 120	134	after 3 months 4.5, 3.6, 3.9, 5.2, 3.3, 3.8	4.1
6	18	4	80, 100, after 7 months 65, 65, 70, 59, 90, 75	75	1.7, 4.3	3.0
7	19		75, 75, 90	80	7.5, 5.6, 5.2	6.1
8	20	4	130, 130, 75, 45, 130	102	4.1, 2.8, 4.7, 2.7, 3.7	3.6
9	20	4	125, 130, 130, after 1 1/2 year 45	108	2.8, 3.4, 5.0	3.7
10	24	9	245, 245, 113, 130	183	4.0, 5.0, 4.5, 2.7	4.1
11	29		150, 200, 200, 225, 120, 200, 100, 85, 100	153	8.0, 3.0, 2.5, 4.0, 5.0, 4.5, 3.0, 3.0, 4.0	4.1
12	35		225, 75, 160, 55, 110, 40, 250, 225, 300, 475, 400, 300, 275 after 2 yrs 50, 80	201	6.0, 5.0, 6.0, —, 3.0, 0.5, 0.5, 4.5, 3.5, 1.0, 5.5, 4.5, 3.5, 5.0, after 2 yrs 2.2, 5.0	4.3
13	39		110 after 1 year 110	110	4.4, after 1 year 3.9	4.2
14	43		160, 150, 193, 138, 220, 110, 210, 225, 250, 250, 250, 250, 210	201	3.7, 5.0, 3.6, 1.5, 4.5, 4.2, 6.0, 3.6, 9.0, 6.0, 4.0, 7.2, 5.5	4.9
15	46	5	245, 55, 55	118	3.7, 1.1, 3.2	2.7

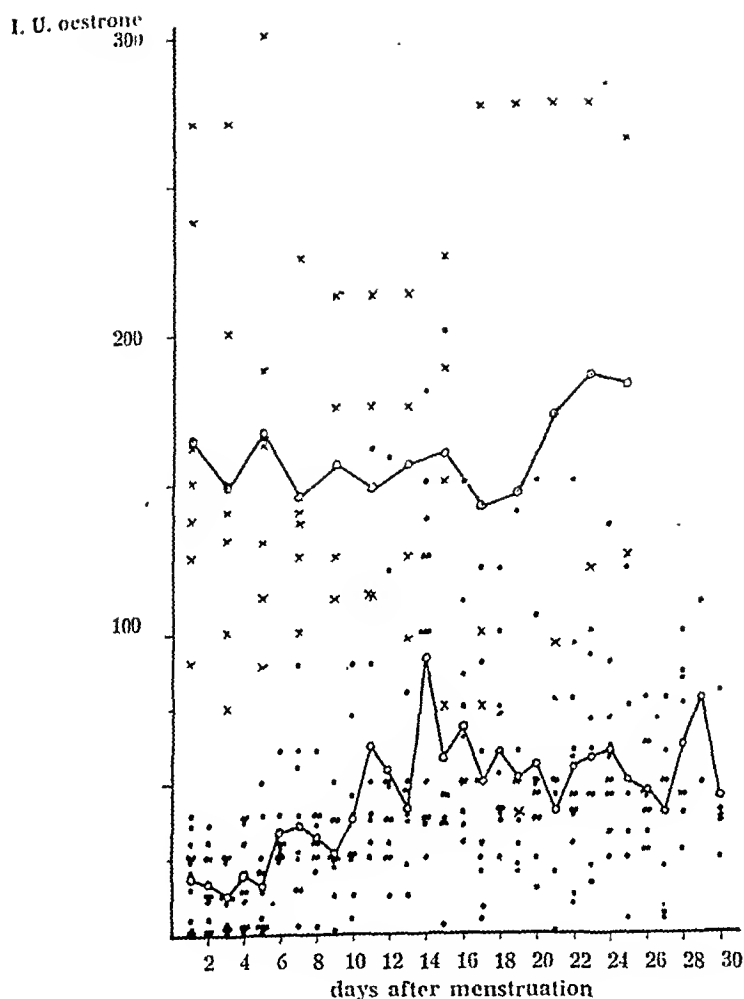


Fig. 1.

Excretion of oestrogenic substances in the urine in healthy women (• ○) and in cases of metropathia haemorrhagica (× ○).

much greater amounts of oestrogenic substances are inactivated than under normal conditions. With the exception of case 5 in all the cases it was possible to produce the desired effect, which resulted in cessation of oestrogenic excretion (fig. 2).

As it can be seen from fig. 2, the level increased after various intervals. The fact, that the excretion did not decrease or increased again after a period may be interpreted by the detachment of the omental flaps from the ovaries. This could be verified in cases nos 5 and 12, at a hysterectomy performed later.

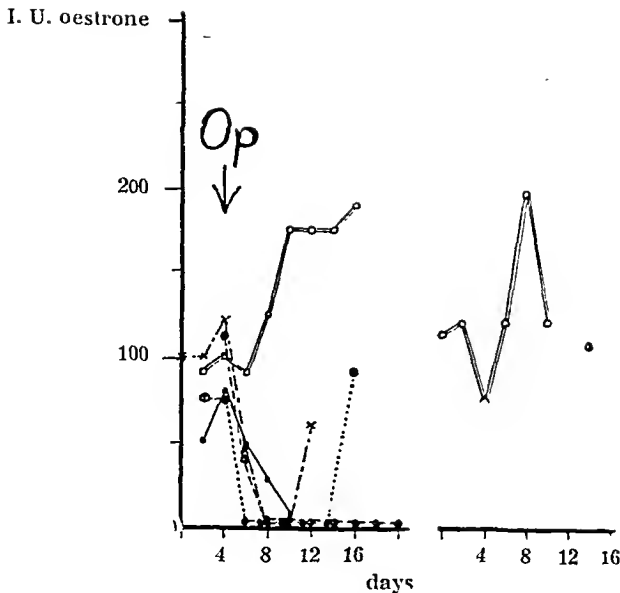


Fig. 2.

Excretion of oestrogenic substances in the urine before and after omental implantation in the ovaries in patients with metropathia haemorrhagica.

The ovaries were examined histologically in four cases (Nos 4, 7, 12 and 13). In two of these patients (Nos 4 and 7) the ovaries showed hazelnut-sized follicular cysts. Case 12 exhibited cystic degeneration of the ovaries. Case 13, on the other hand showed no cysts at all. Only a few primordial follicles and corpora albicantia were to be found. The stroma was fibrous and very poor in cells. None of the ovaries examined contained corpora lutea. It is worthy of note, that the oestrogenic excretion in Case 13 both immediately before the operation and one year thereafter was just as high as in the other three cases with cystic ovaries.

Group 3. Patients treated with prominal.

Five of the patients (Cases 2, 3, 6, 8 and 15) were treated with prominal in doses of 1 gm three times daily for one week. The principles of this treatment were presented by *Westman* in 1943. The purpose of the treatment was to suppress an assumed state of excitation in the hypothalamus, comparable

with the effect produced in exophthalmic goiter. (Falta, 1937). This treatment resulted in a considerably decreased excretion of oestrogenic substances during the period of drug administration, but it increased again to the previous levels a few days after the completion of the treatment (fig. 3).

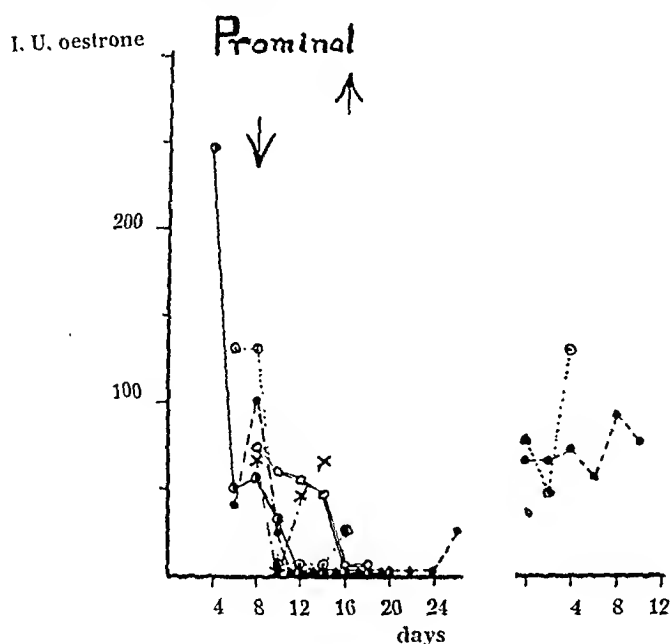


Fig. 3.

Excretion of oestrogenic substances in the urine of cases of metro-pathia haemorrhagica treated with prominal.

Determinations of 17-ketosteroids in the urine.

As stated above, the determination of 17-ketosteroids was carried out in the same sample of urine used for titration of oestrogenic substances. The urine of all the patients included in this material was checked for 17-ketosteroids. The results illustrated in figure 4 show that the levels are consistently lower in these patients than in 19 healthy women. Despite the fact, that the oestrogenic excretion was influenced both by omental implantation and by the prominal treatment, the 17-ketosteroid excretion after both these treatments remained unchanged at the same low level.

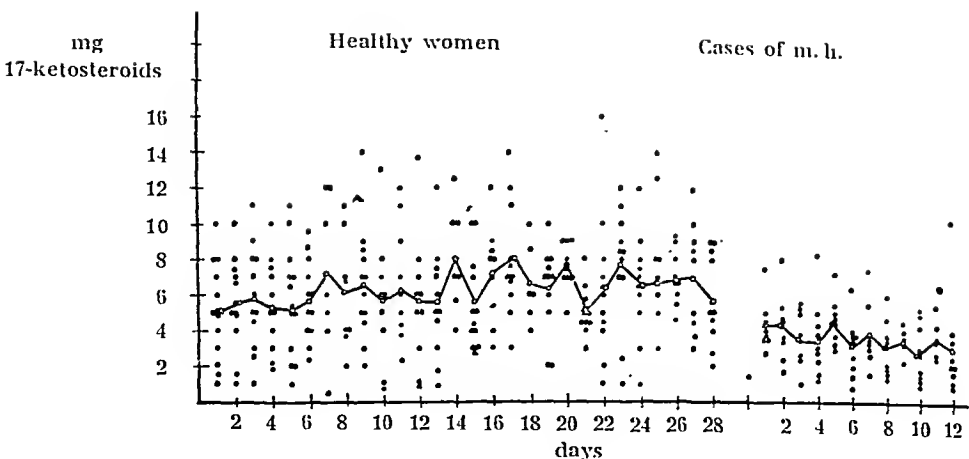


Fig. 4.

Urinary excretion of 17-ketosteroids in healthy women and in cases of metropathia haemorrhagica.

DISCUSSION

All the patients suffering from metropathia haemorrhagica showed a considerably increased production of oestrogenic substances, which was manifested in an increased urinary excretion. This result is in accordance with the findings published by *Genell* (1941). *Genell's* observation also conforms with the present study, that the levels vary, with the difference, that the lowest levels in the present material are relatively high compared with the normal curve. (Fig. 1.) The consistently high content of excreted oestrogenic substances is surprising in view of the varying appearance of the ovaries. Three distinct types of ovaries are represented in this material, i. e. ovaries with follicular cysts, ovaries with small cysts and atrophic ovaries. These findings correlated with the constantly high levels of excretion are indicating, that the increased oestrogenic production of these ovaries is caused by structures, which are not observed at routine gross or microscopic examination. Apart from the cause of the appearance of these three main types of ovarian changes in metropathia haemorrhagica, there is present in all three types a clear gonadotrophic stimulus that leads to an increased production of oestrogenic substances. It cannot be definitely established with the method

used in the present investigation, whether the existence of the three main types means, that the gonadotrophic factor, that produces oestrogenic excretion from the ovary possesses a component with another point of attack in the ovary, or is caused by purely pathologic processes in the ovary. This point, however, must be regarded as of secondary importance, since the essential question appears to be to decide which factors are responsible for the increased oestrogenic production under these pathologic conditions.

Understanding of the oestrogen-producing structures in these ovaries and of the conditions under which they are produced, might reasonably be expected to throw light in the problem of whether these glandular cells develop as a direct consequence of a changed pituitary body stimulus or the damage can be traced to the ovary itself.

A method for direct analysis of the oestrogen-forming structures in the ovary in the rabbit, the mouse and the guinea pig was evolved by *Claesson & Hillarp* (1947) and has been applied in modified form in human beings. (*Claesson, Hillarp & Johansson*, 1948). The increased urinary excretion of oestrogenic substances in metropathia haemorrhagica has been reduced considerably by means of omental implantations in the ovaries and by the action of prominal treatment on the hypothalamico-hypophyseal system. Under the former conditions the oestrogenic substances are transformed into inactive form through the liver. In this way the depressive substances is eliminated. No definitive results of this treatment have been secured so far however, due to the abovementioned technical difficulties.

The picture with regard to the prominal treatment is different. Prominal has a definitely sedative action on the hypothalamus. Therefore, since *Westman, Jacobsohn & Hillarp* (1943) established the nervous communications between the anterior lobe of the hypophysis and the hypothalamus, the results seem to indicate that the hypothalamico-hypophyseal system is in a condition of abnormal excitation, caused either by the oestrogenic action on the hypophysis or by a primary disorganization of the said system. The results of the present

experiments with prominal therefore appear to leave open the question, whether the primary injury is situated in the ovary or in the hypothalamico-hypophyseal system.

The correlation between the amount of oestrogenic substances in the urine and the age of the patient which *Genell* established in 1941, does not seem to fit into the foregoing discussion of the pathologic interplay between the ovary and the pituitary body. For *Genell* interprets the correlation as due to an external action on the ovary, thus disregarding the possibility that also in the upper age group the pathologic condition is to be attributed to the hypothalamico-hypophyseal system, which is the dominant factor in the interplay. Proceeding from this, the correlation between age and oestrogen excretion cannot be regarded as relevant. Despite the vaguely formulated discussion presented by *Genell* his results appear to be indisputable. Consideration of the present material on the same principles reveals fundamentally similar results (fig. 5).

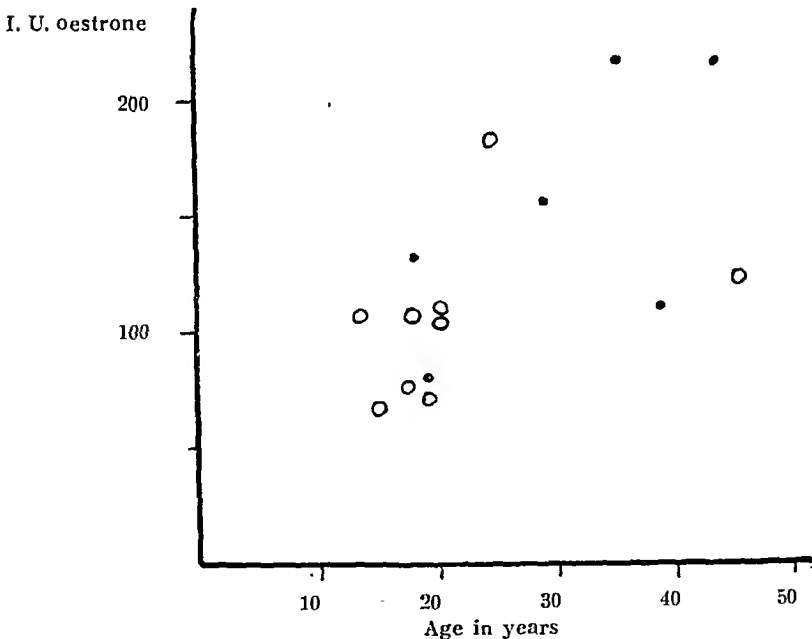


Fig. 5.

Relation between oestrogenic excretion in the urine in cases of metropathia haemorrhagica (mean value per case) and the age of the patients. ○ = cases in which the duration of the disease could be ascertained.

The women in the upper age groups usually had had the disease longer than the young girls. The duration of the disease could be established in 9 cases. The diagram in figure 6 shows the urinary excretion of oestrogenic substances compared with the duration of the disease in these cases. As appears from

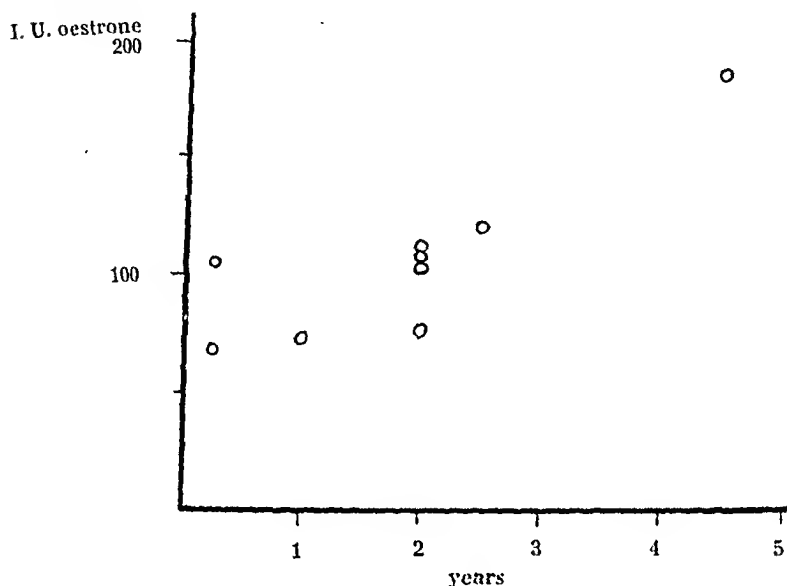


Fig. 6.

The relation between oestrogenic excretion and duration of the disease.

this diagram the amount of the excretion increases with the duration of the disease. Therefore the correlation observed between the patient's age and the oestrogenic excretion may possibly be due to the fact that the older patients had had the disease longer than the young ones. This question should be investigated. Studies by *Claesson, Hillarp & Johansson* (1948) make it appear probable that the production of oestrogenic substances normally takes place in the theca interna. An investigation on the conditions in metropathia haemorrhagica is planned, and the question whether the oestrogen-producing glandular mass increases in this disease will be of special interest.

In conclusion it may be said that the present investigation leads directly to the assumption that the hormone-producing

elements in the ovary are an integral factor in the pathophysiology of metropathia haemorrhagica. This study does not, however, yield an explanation of the origin of the disease or of its course. A number of workers have succeeded in producing experimental metropathia haemorrhagica in guinea pigs by means of resection of the ovaries. This procedure provides an opportunity to analyze the effect of the disease on the ovarian remnants left in place. By applying the histochemical method described by *Claesson & Hillarp* (1947) it should be possible to elucidate the specific ovarian changes with regard to the formation of oestrogen in this disease, which is produced experimentally by a primary ovarian injury. An investigation along these lines is being conducted at present.

The increased production of oestrogenic substances in metropathia haemorrhagica probably leads to damage to the pituitary cells comparable to that which occurs in animal experiment with administration of oestrogenic substances over a long period of time. Hence the constantly decreased excretion of 17-ketosteroids may perhaps find its explanation in the circumstance, that the pituitary function is pathologically changed.

SUMMARY

The total urinary output from 15 patients suffering from metropathia haemorrhagica was collected daily. The excretion of oestrogenic substances and of 17-ketosteroids was determined in the same samples of urine. The same method was used as in a similar investigation on 19 healthy women. All the present 15 patients exhibited the classical picture of metropathia haemorrhagica. The material is divided into three groups. Group 1 includes the patients treated with curettage only. The patients in Group 2 were treated with implantation of omental flaps in both ovaries, while those in Group 3 were given treatment with prominal 1 gm three times daily for about a week. The excretion of oestrogenic substances was considerably higher than normal in all the cases, and no decrease could be observed during the bleeding periods. Follow-

ing omental implantation the oestrogenic excretion ceased and then increased after varying periods of time. Oestrogenic excretion also ceased during treatment with prominal, but returned to the premedication level a few days after completion of the treatment.

The oestrogenic excretion appears to increase with the duration of the disease.

The excretion of 17-ketosteroids was considerably lower than in healthy women. Neither omental implantation nor prominal treatment had any effect on this excretion.

The pathogenesis of metropathia haemorrhagica is discussed in light of the results of this study. An experimental investigation on guinea pigs has been instituted in order to discover which structures in the ovary are responsible for the increase in oestrogenic excretion.

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*Announcements from
the Endocrinological Societies*

DANISH SOCIETY FOR ENDOCRINOLOGY

9. Meeting, April 27, 1948, Zoölogical Museum, Copenhagen.

Rolf Meier (Basle): Conditions of optimal effect of steroid hormones.

NORWEGIAN SOCIETY FOR ENDOCRINOLOGY

1. Meeting, April 22, 1948, State Hospital, Oslo.

O. J. Malm: Parathyroid adenoma (Biochemistry).

Johs. Hagtvædt: Parathyroid adenoma (Internal Medicine).

K. Liavaag: Parathyroid adenoma (Surgery).

From Drammen Hospital (Norway), Surgical Department
(Chief: Knud Nicolaysen, M. D.) and Medical Department
(Chief: Olaf Römcke, M. D.)

CARCINOMA OF THE PARATHYROID WITH HYPERPARATHYROIDISM

BY

BJARNE FRETHEIM and HENRIK F. LANGE

Since the Swedish anatomist *Sandström* in 1880 discovered the parathyroid glands many cases of tumour, both benign and malignant, in these glands have been reported, for the first time probably by *Erdheim* in 1903, who found an adenoma in a parathyroid. These tumours first acquired practical interest when *Mandl* in Vienna in 1925 removed a parathyroid adenoma from a patient suffering from hyperparathyroidism. The first important work on primary hyperparathyroidism came from *Fuller Albright* and coworkers in Boston in 1934. In 1947 *Norris* collected 322 cases of parathyroid adenomas from the world literature. It may be debatable whether some of these tumours ought to be reckoned as hyperplastic or as hypertrophic growths.

Here in Norway the parathyroid adenoma was first described by *Francis Harbitz* in 1915, and the first operation for hyperparathyroidism due to parathyroid adenoma was performed by *H. Fr. Harbitz* in 1936. In 1943 *A. Schrumpf* assembled the 38 cases which up to then had been operated in Scandinavia.

Cancer originating from the parathyroids has extremely seldom been reported. In some of the reported cases the diag-

nosis seems to have been quite uncertain, partly due to lack of definite proofs to show that the tumour really was malignant, partly due to uncertainty whether the malignant tumour had its origin in a parathyroid gland.

Three undoubted cases of primary parathyroid carcinoma with hyperparathyroidism are described earlier (*Black*, 1948, *Gentile et al.*, 1941, and *Meyer et al.*, 1939).

In addition eleven other cases from the literature have been found (*Alexander et al.*, 1944, *Burk*, 1947, *Fasiani*, 1923, *Guy*, 1929, *Hall & Chaffin*, 1940, *Price & Mowatt*, 1932, *Petersma*, 1937, *Quick et al.*, 1931, *Sainton & Millot*, 1933, *Snell*, 1936, *Wellbrook*, 1929). In some of these, hyperparathyroidism is undoubtedly present, but there seems to be no certain evidence that the parathyroid tumour found is a genuine carcinoma, while in other cases the tumours seem undoubtedly to be carcinomas, but there are no definite signs to show that they originate from the parathyroids.*)

In order to establish that a tumour is a malignant growth originating from the parathyroid gland it must be demanded that the tumour presents indisputable signs of malignancy, and not merely uncertain signs observed on histological examination. Or, if the malignancy is incontestable, one must demand that the tumour can with certainty be said to arise from a parathyroid gland. Here there will be abundant opportunities for errors. In the former case because it will often be difficult, on microscopical examination alone, to draw the boundary between simple adenoma and »malignant adenoma«. In the latter case the differentiation will sometimes be still more difficult, if signs of hyperparathyroidism are absent. What will be particularly likely to lead to errors is cancer originating from aberrant thyroid tissue or from the glomus caroticus, or more rarely, cancer metastases from other organs, especially hypernephroma metastases, whose histological pic-

*) In a recent publication *Norris* has from the »the world literature« assembled 15 certain and 3 dubious cases of carcinoma of the parathyroids, 7 of whom had hyperparathyroidism. Most of these cases we have described as not quite certain parathyroid carcinomas.

ture may be confused with cancer of the parathyroid gland. When to this is added the fact that in case of simultaneous metastases of cancer to the skeletal system one may find hypercalcemia and increased tendency to formation of renal calculi, as well as hypercalcuria and increased serum phosphatase, the differentiation may be very difficult. In case of metastases to the bones, however, we do not find hypophosphatemia. Another point of guidance is the typical postoperative reaction seen after removal of a tumour causing hyperparathyroidism.

In the Medical and Surgical Departments of Drammen Hospital we have seen a case of hyperparathyroidism which seems to have been due to cancer of the parathyroid gland.

CASE RECORD

The patient was a chauffeur, aged 40, who had been in good health until the autumn of 1943. He then began to be troubled by pains and stiffness in the joints. It was regarded as »rheumatism« and he was treated with injections of gold. This treatment was discontinued in July 1946, as he began to suffer from symptoms of dyspepsia. Gradually he became weak, got dyspnea and his general condition grew worse.

On 31/10 1946 he was admitted to Drammen Hospital, Medical Dept., as his doctor had found a swelling in the patient's jugulum, and believed that there existed either a malignant tumour or a disease of the blood. The immediate impression given by the patient was that he was suffering from a malignant disease. He seemed very weak and his complexion was pale and yellowish. To the right in the jugulum there could be felt a tumour hardly as large as a walnut, hard, nodular and rather firmly attached to the surroundings. It moved slightly when he swallowed. At first sight it was suggestive of metastasis from a malignant tumour.

X-ray examination of thorax, oesophagus and stomach showed normal conditions. As the feces constantly gave positive benzidine reactions, X-ray examination of the colon was

made. As a incidental discovery was found severe bilateral nephrocalcinosis. On the basis of this finding there was then recorded the typical picture of hyperparathyreoidism, with hypercalcemia, hypercalcuria, hypophosphatemia, increased phosphatase activity, osteitis fibrosa generalisata with osteoporosis in the skull, in the ribs and in the bones of the extrem-



Fig. 1.

Section through parathyroid tumour, firmly rooted in the capsule of the thyroid gland.

ities. In addition was found, as secondary consequence of the nephro-calcinosis: isosthenuria, azotemia and acidosis.

On the diagnosis of parathyroid tumour *hemistrumectomy dextr. and extirpatio tumoris (Nicolaysen)* were performed on Nov. 28, 1946. The tumour was seated in the lower lateral pole of the right thyroid gland. It infiltrated the surrounding muscles of the neck, the deep cervical fascia and the capsule of the thyroid, and caused lateral displacement of the carotid artery. The tumour was removed radically together with the right lobe of the thyroid. It was also found necessary to remove the recurrent nerve. The left lobe of the thyroid had a quite normal appearance and no parathyroid adenoma was found therein (Fig. 1).

The tumour was the size of a pigeon's egg, of irregular shape and nodular surface and of rather hard consistence, without any sharp delimitation. The cut-surface was of greyish-white colour, was very firm in some parts and soft, almost ramollissent in other parts. At the line of transition to the thyroid gland there was seen a 1 to 1½ cm. wide zone with ochre-yellow areas imbedded in lime. Besides there was found a particle of tissue resembling a normal parathyroid. The thyroid gland was of normal appearance.

The microscopical examination (Kreyberg) revealed: »Car-



Fig. 2.

Microphotograph of cancer of the parathyroid gland. (Infiltrating growth of strips of a slightly differentiated epithelium in solid heaps without sharply defined cell boundaries. The nuclei are fairly large, roundish-oval in shape with finely distributed chromatin. Some mitoses are seen. The strips of epithelium are separated from each other by slender strands of connective tissue, poorly supplied with cells). $\times 100$.

cinoma, not much differentiated. The picture is quite compatible with the assumption of parathyroid origin.» (See fig. 2). The small piece of tissue showed: Normal parathyroid. (See fig. 3).

In the first few days after the operation the patient was

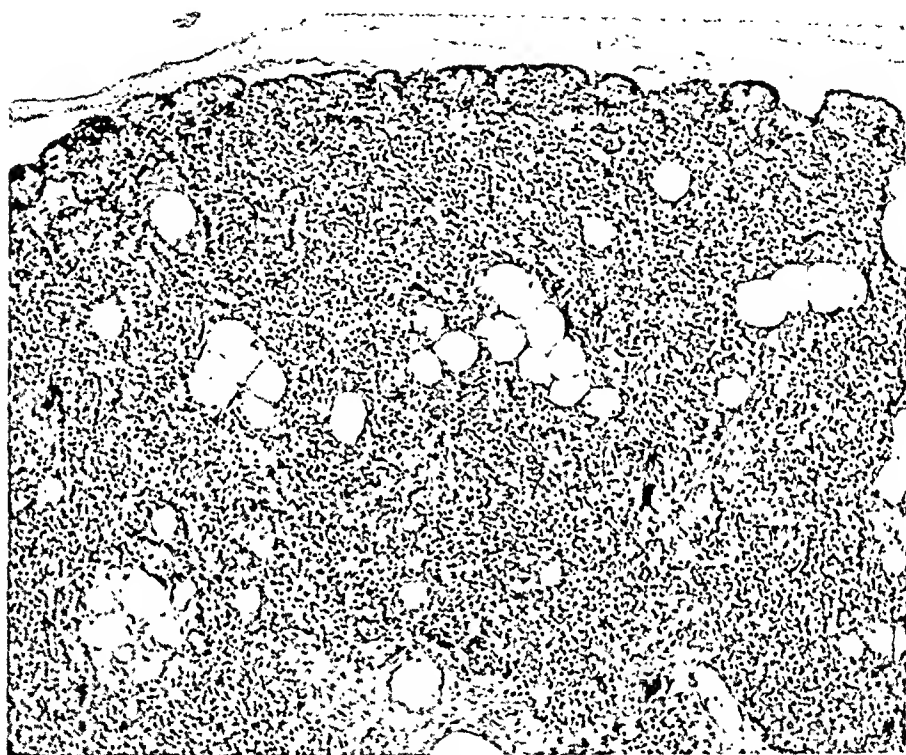


Fig. 3.
Normal parathyroid. ($\times 100$).

troubled by a sensation of bodily unrest and slight paresthesia in hands and feet. The feeling of unrest was at times so great that he could hardly sleep, but it disappeared for shorter periods. There were signs of latent tetany, which became manifest on the 7th day after the operation, with stiffness especially in the fingers and around the mouth and with distinctly positive Chvostek and Trousseau phenomena. At the same time the calcium content in the serum had fallen from 18 mg. per cent on the day of operation to 7.5 mg. per cent (fig. 4).

During the afternoon the patient was given 100 drops of AT 10 per os. The sensation of stiffness gradually subsided during the evening, but came on again in the night. On the next day he was again given AT 10, as well as calcium lactate, and the manifest tetany once more disappeared, while he con-

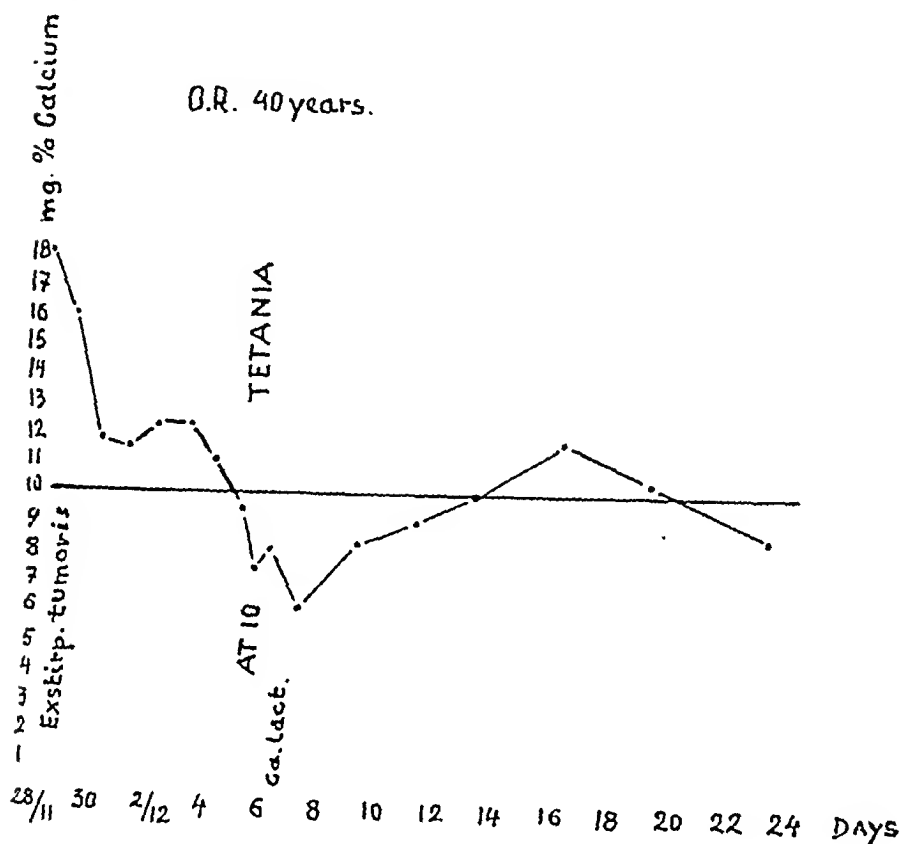


Fig. 4.

Serum calcium in mg. per cent. before and after extirpation of tumour.

tinued to show signs of latent tetany. The sensation of stiffness in the fingers and the feeling of unrest did not disappear until about five weeks after the operation, at which time the serum calcium became stabilized at about normal values.

The other laboratory findings (Table 1) show that the serum phosphorus rose somewhat, to low normal values. The serum phosphatase fell somewhat, but was still too high at the

Table 1.
Laboratory findings before and after extirpation of tumour.

	Nov. 1946	Operation	Dec. 1946	Jan. 1947	Feb. 1947	April 1947	Jan. 1948	Various examinations.
Sediment. rate, mm.	17-25		120	93-45	15	16	10	R. bl. c.: 5.2 mill/mm ³
Hb. %	91		75	86	93	97	105	W'h. bl. l.: 9000-10000/mm ³
Weight, in kg	58		64 64		66	65	67	M. K. R. ÷ Mantoux ÷
Proteinuria	—	+	tr. — tr. tr.		tr.	tr.	tr.	Sternal Marrow: normal
Urine, sp. gr.	1008-1010		1010	1010	1010	1010	1010	Rectal examination:
Urea clear. %						20	20	neg. findings
Blood pressure	110/70				140/100 150/100	180/100 180/105	150/90 195/120	Rectoscopy: neg. findings
Ophthalmoscopy	normal					Normal		Ewald: Achlorhydria
Ur +, mg %	100-58	110	165 80 48	49		6.5	6.5	
Total prot. g %			5.7					
Alk. res. vol. %		40	45 63 50 45 57		49	54	50	
Phosphatase, (Bodansky)	13.6		9.0				23	
Phosphorus, mg %	2.7	2.9	2.5 2.1 2.0 3.1				3.3	
Serum colour	9-6						8	
Ur -, mg %			12.3				11	
Fig.: QT/freq.	0.30/72	0.48/66				0.48/48	0.44/54	
Calcium, mg %	16.4-14.8		See figure 4	5.6-9.8	9.8	10.6	10.6	

time of discharge from hospital. The non-protein nitrogen in the blood sank towards normal value and the alkali reserve rose to the normal figure, while isosthenuria persisted. On the

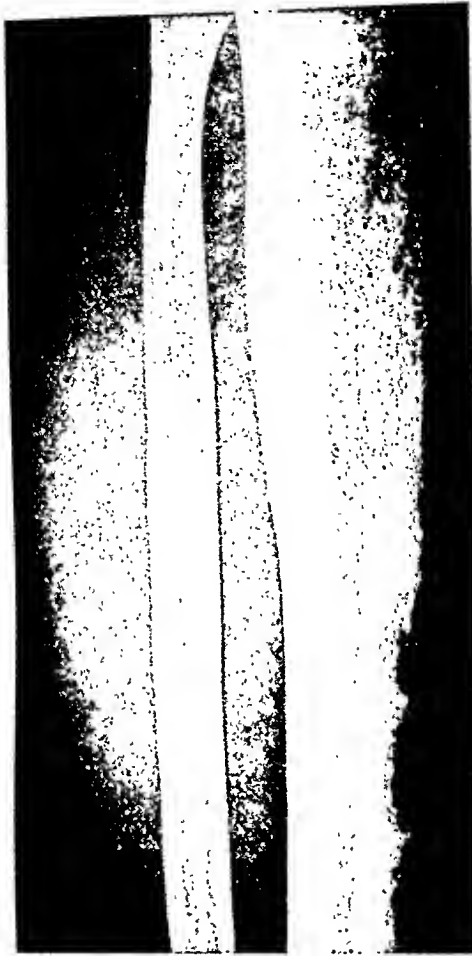


Fig. 5.

Cystic radiolucencies in the left tibia and fibula before the operation.

third day after operation he got symptoms resembling those of rheumatic fever, with pains and swelling in both ankles and in the right wrist. At the same time the sedimentation rate rose to 120 mm. He was given salicylates and in the course of 8 to 10 days the swelling in the joints subsided, but the pains persisted all the time he was at the hospital. His voice

remained hoarse after the operation, but improved gradually. During this time the substitution therapy was readjusted from AT 10 to D₂-oil and he was discharged on 22/1—1947 with a daily dose of 10 drops of D₂-oil.



Fig. 6.

The radiolucencies in left tibia and fibula have almost disappeared one year after the operation.

The patient has since been to the hospital three times for control examination. A steady improvement has been observed. The pains in the joints disappeared, his strength increased and he resumed his work as chauffeur four months after the ope-

ration. On his own initiative he ceased taking the D₂ drops in July 1947, but the improvement persisted without change. At the last control examination in January 1948 he presented a healthy appearance, he felt well and there was no sign of local recurrence. Calcium, phosphorus and phosphatase content in serum were normal, but renal insufficiency persisted, with

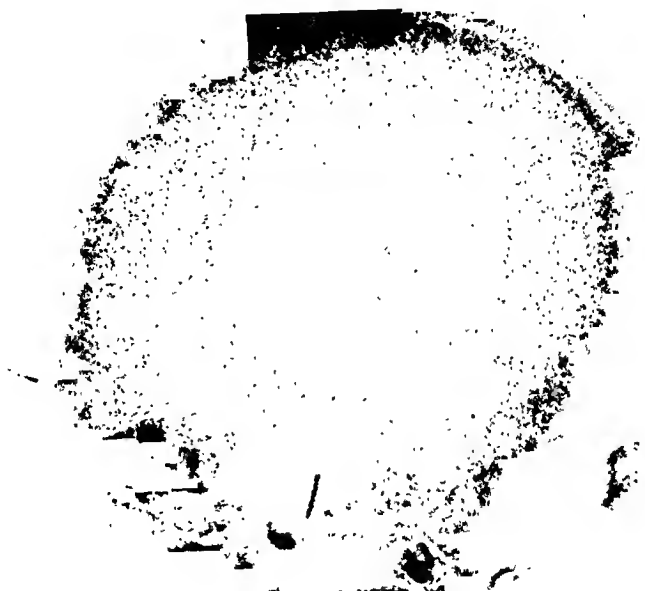


Fig. 7.

Cystic radiolucencies in the cranium before the operation.

isosthenuria, N.P.N. 86 mg. per cent and alkali reserve 50 vol. per cent. While the blood pressure before the operation was normal, it was found to be persistently increased after the operation.

Very remarkable is the improvement noted on radiographic examination of the bones (fig. 5, 6, 7 and 8). The radiolucent areas in the left tibia and fibula are seen to be considerably reduced and those in the cranium have practically disappeared.

The calcifications in the kidneys, however, persist unchanged.

DISCUSSION

We believe that the tumour here described is undoubtedly a parathyroid carcinoma accompanied by hyperparathyroidism. This view is in accordance with the pathologic-anatomical findings and with the typical post-operative reaction noted. The further course of the illness seems to indicate that, at

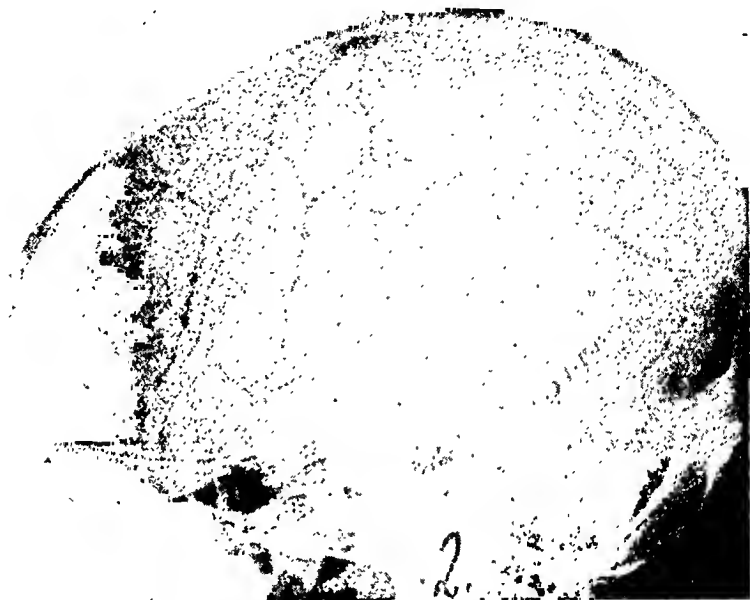


Fig. 8.

The radiolucencies in the cranium have practically disappeared one year after the operation.

any rate up to the present there has come no recurrence or metastasis. The normal appearance of the parathyroid gland, both macroscopically and microscopically, would not likely be found in case of a malignant tumour of an other origin accompanied be secondary hyperparathyroidism.

The renal function has not improved after the operation, however, and we can here hardly count upon any improvement in the future either, so that the prognosis in this respect is relatively unfavourable.

A peculiar phenomenon, which perhaps bears some relation to the affection of the kidneys, is the hypertension that seems to have developed after the operation. No definitive explanation of this circumstance can here be given, but it is quite possible that the normality of the blood pressure before the operation was only apparent, being a consequence of the general hypotension observable in hyperparathyroidism.

Another circumstance of which we can likewise give no definite explanation is the occurrence of symptoms of acute articular rheumatism. There may possibly have here existed an atypical form of rheumatic fever, but it can hardly be deemed out of the question that the phenomena may in one or other manner be connected with the hyperparathyroidism. At any rate, it is remarkable that they developed together with the symptoms of hyperparathyroidism and disappeared simultaneously with the improvement in that condition. Especially remarkable is the acute aggravation of the rheumatic symptoms after the operation, with marked rise in S.R., which afterwards gradually fell again to the normal.

SUMMARY

There is described a case of primary parathyroid carcinoma with hyperparathyroidism. One year after radical extirpation of the tumour no signs of recurrence or metastases were noted.

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DEMONSTRATION OF SUBSTANCES WITH OESTROGENIC EFFECT IN HUMAN SPERM

BY

OVE RIISFELDT

The chief object of the transfer of sperm to the vagina is that of bringing the spermatozoa into contact with the female internal genitals, in order that they may impregnate the ova. However, the question has been considered whether the sperm may also otherwise influence the female organism, more particularly whether a resorption may take place from the vagina of the substances contained in the sperm plasma.

First the possibility suggests itself that the coitus may elicit morphological changes in the female organism. It is a well-known fact that in rabbits coitus is attended by ovulation. Yet, in these animals ovulation can be brought about also by an electric irritant, for instance.

In the woman there occurs *as a rule* regular cyclic ovulation. A few writers, e. g. *Bolaffio* (1933) and *Wittenbeck* (1930), claim to have observed ovulation in connection with coitus. No definite conclusion can be drawn, however, from such an observation, because two or more ovulations may very well take place within one cycle, so accordingly the possibility cannot be excluded that the ovulation observed would have occurred also without the attending coitus.

Things are different where the resorption of substances

from the sperm is concerned. It is a fact that resorption may take place through the vaginal mucous membrane. Recently *Goldberger, Walter & Lapid* (1947) showed that penicillin can be resorbed from the human vagina.

Green-Armytage (1943), on the basis of clinical as well as experimental observations, claims to have demonstrated changes in the female organism which he refers to vaginal resorption of hormonal substances present in sperm. His clinical material consists of two groups, each comprising 20 married women. In group 1 the married life was not started with taking of contraceptive measures, while such were taken throughout in group 2. The gynaecological examination made at the beginning of the married life revealed small uteri in the majority of the women of both groups. After 4½ to 7 months of married life the uterus was found to have grown to the normal size in 9 out of 15 women of group 1, while after 2 years only 5 women in group 2 presented a normal-sized uterus. This difference in the development of the uterus is in *Green-Armytage's* opinion due to resorption of hormonal substances from the sperm in the group where contraception was not applied.

Green-Armytage showed that intramuscular injection of human sperm into infantile female rats caused cellular and glandular hyperplasia together with marked growth of the muscular layer in the uterus. Haemorrhagic follicles were observed in the ovaries. He believes that the changes are due to testosterone, transformed in the female organism to substances with oestrogenic effect, or possibly to a stimulation of the pituitary gland.

Bacsich, Sharman, & Wyburn (1945) tried to test the results of these experiments. They treated 3 out of 6 infantile rats by subcutaneous injections of 0.5 ml of sperm every other day for 14 days, without, however, observing any changes in the uterus. Similar experiments were made with rabbits and guinea-pigs. The rabbits presented no changes, whereas the guinea-pigs showed a slight increase of the size of the uterus, but no definite growth of the endometrium, nor signs of proliferative changes.

Uchigaki (1929), on the other hand, found that injection of human sperm into mice was capable of making 75 per cent sterile in the course of from 1 to 2 months. Histological examination revealed atrophy of the follicles and fatty degeneration of the uterine mucous membrane.

OWN INVESTIGATIONS

Infantile castrated and young non-castrated female rats were applied for the experiments.

The sperm, derived from fertile as well as non-fertile men, was obtained by masturbation, collected in sterile glasses, and stored in refrigerator till it was to be used.

The sperm was in all experiments injected subcutaneously, without having been submitted to treatment of any kind. Neither had it been diluted.

In a few cases several sperm samples could be obtained from the same man. Accordingly a few of the rats could be given sperm from one person during the entire experimental period, whereas the majority had to be given a mixture of sperm samples from different men.

No local reaction, as for instance necrosis, was observed here, unlike what was the case in a few experiments made with the object of studying the contents of oestrogenic substance in equine sperm.

A number of the animals died some time, shorter or longer, after the commencement of the injections. This seems to accord with *Oslund's* (1928) observation of a certain toxicity in connection with the sperm injections. It appeared, however, that the rats into whom sperm from one man had been injected through the entire experimental period did not die, whereas those given sperm collected from different men died in a large number.

1. Sperm injected into infantile, castrated, female rats. In this experimental series each rat was given sperm from one man during the entire experiment. A total of 9 rats were ap-

plied, of which 3 served as controls. 6 were given injections of 0.2 ml of sperm daily. Vaginal smears were taken every day as soon as the vagina had opened. After 19 days all 6 rats responded by an oestrous phase in the vaginal smears. The following day the animals were killed and dissected. There was found a marked increase in the size of the uterus. Microscopy of this organ showed the mucous membrane to be in the phase of proliferation, while a few glands proved to have increased in size, containing more secretion; but the number of glands had not increased in proportion to that in the control animals. Moreover, there was found a far richer vascularisation than in the controls.

2. Sperm collected from different men and injected into infantile, castrated, female rats. 0.25 ml of sperm was injected twice daily into 10 rats. 5 of the animals died already after 3 days, without changes being demonstrable. The remaining 5 were given injections for 21 days. After 2 days the vagina had opened so much that vaginal smears could be taken. An oestrous phase was never demonstrated. Microscopy of the uterus revealed no differences from the conditions in the controls.

3. Sperm injected into young female rats. 14 animals were applied, which were 40 days old at the beginning of the experimental period. 10 of them had 0.2 ml of sperm injected daily for 23 days, after which the vaginal smears showed an oestrous phase in 5. Dissection revealed haemorrhagic follicles in the ovaries, as well as a marked increase in the size of the uterus. The histological changes were analogous to those described in experiment 1. The 4 controls presented no similar changes.

DISCUSSION

According to the above experiments there is no doubt that sperm injected into castrated, female rats is capable in certain cases of eliciting changes in vagina and uterus similar to

those brought about by treatment with oestrogenic hormone. Likewise it appears that sperm contains substances capable of producing haemorrhagic follicles in the ovaries. It cannot be made out from the above experiments whether this latter effect passes by the pituitary body or whether the sperm contains a substance with a gonadotrophic effect. The toxicity of the sperm seems attributable to its contents of proteins.

SUMMARY

It is shown that subcutaneous injection of human sperm into castrated, female rats is capable of bringing about oestrus in some of these. Haemorrhagic follicles can be produced in the ovaries by injection of sperm into young female rats.

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HYPERTENSION PRODUCED WITH DESOXYCORTICOSTERONE ACETATE AND SODIUM CHLORIDE IN A CASE OF POSTURAL HYPOTENSION

BY

ROLF LUFT, GUNNAR SANTESSON and BJÖRN SJÖGREN

In clinical experiments it has been shown by *Perera & Blood* (1947) that DCA (desoxycorticosterone acetate), potentiated by sodium chloride elicits an increase of the original blood pressure in cases of essential hypertension. This increase develops in 2—3 days. In healthy normotensive individuals the blood pressure remains normal or may show a moderate rise after 2—3 weeks of treatment.

The mode of action of DCA in this respect has not yet been fully explained. *Perera et al.* (1944, 1947) could not find any direct relation between the electrolytic changes and the increase of plasma volume to the increase of blood pressure after administration of DCA. The changes mentioned were of the same magnitude in the normotensive as well as hypertensive subjects in spite of the fact that only the hypertensives reacted with a further blood pressure increase.

It is a well established fact that the low blood pressure in adrenal insufficiency is normalized by DCA and sodium chloride (*Perera et al.*, 1944). This form of treatment has been tried by *Gregory* (1945) in a case of »orthostatic hypotension«.

A favourable effect on blood pressure and orthostatic symptoms was obtained. The information given in the article is incomplete and does not elucidate, if the diagnosis of the case reported was postural hypotension or arterial orthostatic anemia.

The aim of the present work is to show the effect of DCA and sodium chloride in a case of postural hypotension.

In postural hypotension the vegetative blood pressure regulating mechanism is impaired due to a lesion in the nervous system (*Ellis & Haynes, 1936, and Stead & Ebert, 1941*). When changing from recumbency to an erect position the circulatory changes induced by the hydrostatic force are not compensated for as normally by the sympathetic nervous system. Normally the result of this compensatory mechanism is maintenance or increase of the diastolic pressure and acceleration of the pulse rate. In postural hypotension standing up leads to a momentary fall of both systolic and diastolic pressure. The pulse rate is unchanged or only slightly accelerated. This »hypodynamic regulatory disturbance« may occur after extensive sympathectomy (*Mac Lean & Allen, 1940*), — operation acc. to *Peel and Smithwick* —, in neurological disorders as tabes dorsalis, syringo- and hematomyelia (*Ellis & Haynes, 1936*), where the sympathetic reflex chain is broken. The symptom complex first described by *Bradbury & Eggleston (1925)* as a clinical entity, also shows other characteristic signs: disturbance of perspiration, a low BMR, impotency, nycturia and gastrointestinal symptoms. In typical cases the disease has developed during several years. It is likely that the central disorder in such cases of postural hypotension is a lesion in the diencephalon (*Ellis & Haynes, 1936, and Stead & Ebert, 1941*). It is important in this connection to differentiate postural hypotension from the more common so called arterial orthostatic anemia, the »hypotonic regulatory disturbance«. In the latter the sympathetic reflex mechanism is functioning normally; no disease of the central nervous system can be proved. When changing from recumbency to an erect position the diastolic pressure is increased and the heart rate accelerated.

CASE REPORT

(No. 234—47. W. E.) Unmarried man, 47 years old. Attack of polyarthritis 1913. Then healthy till 1938, when he began to feel tired and had seizures of dizziness, most pronounced in the mornings and on warm days. The symptoms got worse. Libido decreased, and during the last few years he has been completely impotent. The ability to perspire disappeared. No favourable effect was obtained with sedatives, liver- and iron preparations.

When entering hospital 1946 he continuously felt very tired. Fainted now and then and thereby got hurt several times. At examination he was found to be a pale, tall and slender man. Body length 176 cm, weight 57 kg. Could not keep an erect posture without fainting. No abnormal pigmentations on skin or mucous membranes. No cardiac incompenstation. Neurological examination with normal findings. No reaction to carotis pressure. Secondary sex characters normally developed.

Hemoglobine 70 %, red cells 3,4 mill., white cells 5000. Differential count normal. No albuminuria or glycosuria, sediment normal. WaR in blood and liquor negative. BMR + 1 %, 17-ketosteroids in urine normal. CO₂-combining power 67 volume %. Erythrocyte diameter 8.04 μ . Takata neg. In serum: bilirubin 0.5 mg %, alkaline phosphatase 6 Bodansky units, potassium 20 mg %, sodium 330 mg %, calcium 9.4 mg %, inorganic phosphorus 3.9 mg %, iron 100 γ /ml.

Water test (acc. to Volhard-Strauss, 1000 ml.): 700 ml excreted during the first 4 hours, dilution to sp.gr. 1.005, concentration to 1.023. Inulin clearance 99 ml/min. Diodrast clearance 390 ml/min. Galactose tolerance test: 0.4 g excreted in 4 hours. Sternal puncture: normal marrow.

X-ray examinations of heart, lungs, abdomen (no adrenal calcifications), stomach, intestines, skull, all with normal findings. ECG at rest normal, in erect posture normal with no pulse acceleration, and no changes as those seen in orthostatic anemia.

Blood Pressure. Morning blood pressure, fasting, systolic 90—120. diastolic 50—70 mm Hg, in erect posture systolic 50—60 mm Hg, diastolic not measurable. After Gynergen 0.5 mg subcut. 175/125, in erect posture 100/60 mm Hg. This rise was also obtained with adrenaline and ephedrine, the postural reaction still remaining but not as pronounced.

Fig. 1 shows an orthostatic test performed with the patient. At sudden transition from lying to erect posture the systolic as well as diastolic pressure instantly declines. The pulse rate is unchanged. The patient feels dizzy and faints, without showing perspiration.

If the patient was put on a tilting table the same changes were obtained in slight »head-up« position, while »head-down« position

In this case the diagnosis postural hypotension was quite evident and corresponded in all details with the description given by *Bradbury & Eggleston* (1925). There was a slow progress of symptoms. No signs of adrenal cortical insufficiency: no pigmentations, no adynamia, no gastro-intestinal changes, normal electrolytic balance, almost normal water test, and a duration of the disease of 9 years. The Cutler test was negative. Postural hypotension is not typical in Addison's disease despite of contradictory statements in literature (*Šjögren*, 1947). Arterial orthostatic anemia was ruled out with all evidence. We want to emphasize the normal ECG-findings (*Ewert*, 1938) and the reaction to Gynergen. In postural hypotension Gynergen gives relief of symptoms, in orthostatic anemia deterioration with more pronounced symptoms.

Methods. The patient was not allowed to get out of bed until the blood pressure was determined early in the morning. Blood pressure was checked for several months before treatment. Fluid intake and urine as well as chlorides in urine were measured daily. Hemoglobine, red blood cells and hematocrite value were determined three times a week. Plasma and blood volumes were determined with Evans' Blue according to *Gibson & Evans* (1937). Inulin and diodrast clearance were determined after end of treatment. During the experiment the patient was put on a »salt-free« hospital diet containing about 3 g NaCl daily.

Effect of treatment (fig. 2). DCA was administered with 20 mg daily for 53 days. During the first 35 days NaCl was given simultaneously.

Before DCA- and sodium chloride treatment was started, the patient had a comparatively low systolic as well as diastolic blood pressure and showed pronounced, typical postural phenomena. Fluid intake and urine volumes corresponded to each other. The excretion of chlorides was definitely low (diet containing about 3 g NaCl).

When DCA- and sodium chloride treatment was started, an immediate and rapid increase of the systolic as well as diastolic pressure followed. At the same time the hematocrite values, hemoglobine and red blood cells decreased. The urine

volumes also decreased greatly, in spite of increased NaCl — excretion. The postural mode of reaction was in spite of the blood pressure increase unchanged. But when the blood pressure level was greatly increased, the patient felt very much

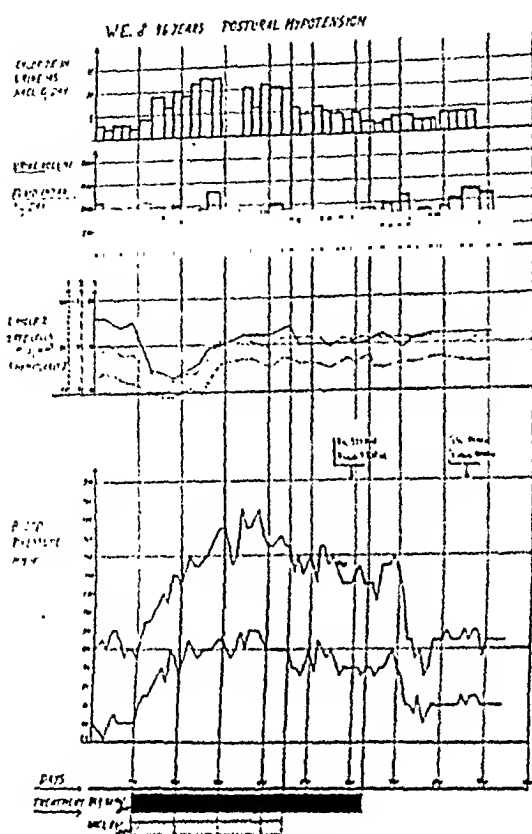


Fig. 2.

Effect of DCA and sodium chloride in a case of postural hypotension.

recovered, could stand upright and move about (see fig. 1). Before treatment the patient was completely unable to work. After administration of DCA for some time, the patient considered himself well enough to begin working.

In 15—20 days after starting DCA- and NaCl-treatment the hematocrite, hemoglobine and red blood cells attained their lowest values, which then increased again. In spite of this the blood pressure continued to rise and reached a max-

imum pressure of about 175/110 mm Hg after 35—40 days. Fluid intake and urine volumes were then approximately equal again.

When DCA-administration 53 days after the beginning of the experiment was interrupted, the blood pressure rapidly decreased to the original level. At the same time the state of the patient grew worse.

The blood volume, measured 49 days after the beginning of DCA-treatment, was 5230 ml. On the 22nd day after interruption of DCA-treatment, it was 4350 ml, consequently a decrease of 880 ml or 17 per cent. Inulin clearance showed a glomerulus filtration of 99 ml/min., renal plasma flow was 390 ml/min.

DISCUSSION

A rapid rise of blood pressure after administering DCA and sodium chloride has earlier only been demonstrated by *Perera & Blood* (1947) in cases of essential hypertension. In our case the same effect has been achieved in postural hypotension.

In our case DCA had a therapeutical effect, yet without being able to affect the postural mode of reaction. It is therefore possible, that the exclusion of the sympathetic regulating mechanism, which causes inability to compensate for hydrostatic blood changes when the patient rises to his feet, also played a part in the rise of blood pressure in this case.

In this special case, there are other factors, which may have been of importance for the blood pressure increase after DCA-administration. Diodrast clearance showed a definitely low renal blood flow. There were, however, no other clinical signs of present or previous kidney disease.

During part of the observation time the patient went through an acute hepatitis. It is well known, that lesions of the liver cells cause changes in fluid balance in the form of an increase of the amount of extracellular fluid and consequently the blood volume (*Lebry & Hoagland*, 1947). By producing greater increase in the blood volume a lesion of the liver cells might affect the blood pressure during the DCA-treatment.

By administration of DCA and sodium chloride we have in our case produced a significant blood pressure increase. At the same time a pronounced decrease of the hematocrite value, the red blood cells and the hemoglobin was obtained indicating an increase in plasma volume. *Perera et al.* (1944, 1947), however, deny that a change in the blood volume could cause the blood pressure increase (in hypertensive patients). In our case, therefore, three factors are present, the importance of which for the rise of blood pressure produced, for the time being cannot be decided: the defective sympathetic regulating mechanism, the lesion of the liver cells and the impaired renal blood flow.

SUMMARY

A case of postural hypotension is described, where the differential diagnosis to arterial orthostatic anemia is emphasized. Administration of desoxycorticosterone acetate (DCA) potentiated by sodium chloride provoked a marked and significant increase of blood pressure and an increased plasma volume. The case was complicated by an infectious hepatitis during the treatment. The diodrast clearance showed a significantly low value. The rôle of the impaired sympathetic blood pressure regulation, the liver disease and the low renal blood flow for the blood pressure increase is discussed.

Desoxycorticosterone acetate together with sodium chloride had a marked therapeutic effect in this case.

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THE SIGNIFICANCE OF HORMONAL
DISTURBANCES FOR THE DEVELOPMENT OF
MENTAL DISEASES*)
(A REVIEW)

BY

A. LETH PEDERSEN

The subject of my address is so comprehensive that I cannot give a proper survey of the conditions within the limited time at my disposal. I shall therefore confine myself to mention some of the most important features and concentrate especially on features in which I myself have taken particular interest.

The neuro-hormonal correlation is very complex, there being a close relationship between the psychic functions, the function of the pituitary-hypothalamic system, and those of the subordinate endocrine glands. These different functions influence each other, so that the least disturbance of one of them will almost inevitable lead to a vicious circle.

It is a well-known fact that purely psychic functions, e. g. emotion, may give rise to endocrine disturbances. Best known is the occurrence of amenorrhea or other menstrual disturbances with various psychic insults and mental diseases. Furthermore, recovery from a psychosis or other mental affection is often seen to be attended by a return to normal hormonal conditions.

*) Address at the meeting of the Danish Society for Psychiatry in St. Hans Hospital, June 9, 1948.

It is, therefore, no wonder that many endocrine lesions are associated with pronounced mental symptoms. The physiopathological conditions are not yet quite clear, but presumably there occurs first a disturbance of the general metabolic processes of the brain, which is then followed by development of a physiopathological activity. Possibly the brain acts as a reservoir for certain hormones (Reiss, 1944).

The endocrine diseases causing mental symptoms are chiefly those occurring in pituitary(-hypothalamus), gonads, and thyroid.

With regard to the *pituitary lesions* there is reason to remark that the pituitary body is so intimately connected with the hypothalamus, both neuro-anatomically and physiologically that they constitute a functional entity. The hypothalamus is, however, supposed to possess an independent secretory activity as well.

Mental symptoms are not a common occurrence in conditions due to hyperfunction of the pituitary body (hyperpituitarism). In conditions due to pituitary hypofunction (hypopituitarism), on the other hand, such symptoms are frequent, particularly so in adiposo-genital dystrophy. This condition being probably due chiefly to a lesion in the hypothalamus, it is reasonable to presume that it is the hypothalamus which is responsible for the mental symptoms in association with pituitary lesions.

The mental symptoms seen in association with hypopituitarism may, according to the literature be either torpidity and indolence, or irritability and emotional instability.

Personally, on examination of a number of patients with pituitary-hypothalamic lesions (Leth Pedersen, 1948), I believe to have ascertained characteristic mental symptoms in the forms of: 1) increased irritability, these patients being emotionally unstable and hot-tempered to the point of explosion, and therefore frequently at variance with their associates; 2) emotional instability, the patients being sensitive with fluctuating moods and a tendency to low spirits; 3) ego-

centricity, the patients being self-pitying, preoccupied about themselves and their disease, inert and despairing, sluggish, self-willed, and hard to please. In my material these symptoms were observed partly in patients with different forms of hypopituitarism, and partly in young women with a characteristic clinical picture, presumably caused by a hypothalamic insufficiency. This picture corresponds exactly to that described by *Bartels & Hjorth* (1947) and designated by them as hypogonadism. The disease occurs exclusively in women within the former half of the sexually mature age, and it manifests itself by gain in weight and the development of a characteristic »healthy, flourishing« appearance. The basal metabolic rate is low, the menses scanty, the libido decreases, and the patients develop the mental symptoms just mentioned, generally attended by tiredness, headache, and different vasomotor disturbances.

Lesions of the *gonads* are far more frequent among women than among men. I shall here concentrate on the conditions in women.

It is a well-known fact that mental symptoms are of frequent occurrence at the menopause, when a gradual involution of the ovaries takes place. There seems to be no doubt that these mental symptoms, characterized particularly by emotional instability and increased irritability, are released by the ovarian insufficiency, but we are not quite clear as to the physiopathological conditions.

Emotional disturbances are seen also during menses, in the form of dysphoria or the like, the cause of which is not evident either. The possibility has been suggested of a connection with disturbance of the water metabolism due to an increased production of steroids (*Hemphill*, 1944). Similar mental disturbances may be met with during pregnancy and puerperium.

»Menopausal« symptoms are more pronounced in women after castration, whether by X-rays or operation. I have personally (*Leth Pedersen*, 1944) examined over 50 women (observation periods up to 20 years) after removal of both ovaries

at the sexually mature age (from the menarche till the age of 40). All these women had hot flushes and also mental symptoms of some kind or other.

The mental symptoms occurring were as follows: 1) disquiet and restlessness. 2) increased irritability with emotional instability and irascibility. These 2 groups of symptoms were present in well over half of the examined women. 3) change of mood in the direction of instability and a tendency to low spirits, likewise found in well over half of the women. 4) impaired memory and failing power of concentration, occurring in ab. 50 per cent. 5) fits of palpitation, dyspnoea, and anxiety, likewise ascertained in ab. 50 per cent. In consequence of these symptoms the ability to work was more or less reduced in a scant 50 per cent of the examined women. The mental symptoms were found to be most conspicuous in the patients with the most pronounced hot flushes, a symptom which, in my opinion, may to a certain extent be taken as a measure for the degree of ovarian insufficiency. I, therefore, believe the ovarian insufficiency to be the cause also of the mental symptoms. This view is supported by the fact that the mental symptoms improve in all cases after large doses of oestrogen and are likely to disappear entirely if only oestrogen is administered in sufficient doses.

Nearly all the female castrates whom I have examined made an energetic and diligent impression, and rather minimized their symptoms, being by no means egocentric, inert, self-pitying, despairing, or preoccupied about themselves and their disease. They differ by this feature and by their restlessness from the women with hypothalamic insufficiency. In my opinion the two different conditions of hormonal insufficiency are attended by distinct and characteristic mental pictures.

Mental symptoms are conspicuous also in association with *thyroid lesions*, presumably because of the effect of these lesions on the hypothalamus. Thus, in the case of hyperthyroidism there are found signs of sympatheticotonia with emotional instability and increased irritability (*Reiss*, 1944),

whereas hypothyroidism is attended with a reduced sympathetic tone with slow reactions and little affectivity.

Adrenal lesions are not attended by constant or characteristic mental symptoms. But attention has for many years been focussed on the adrenals as the possible centre for the pathogenesis of schizophrenia and certain neuroses, especially neurasthenia, probably because these cases somatically resemble the cases with adrenal insufficiency.

It is a fact that there is found a greater proportion of mental cases among dyscrinuous patients (notably among those with lesions of pituitary body and ovaries) than among patients without endocrine diseases. Hence there must be a certain relationship between dyscrinism and mental diseases, particularly schizophrenia. However, no psychosis has so far been established to have a purely endocrine aetiology (*Hemphill, 1944*).

As regards *schizophrenia*, nothing certain is known as yet of the pathogenetic importance of the adrenals. The facts that schizophrenic women are far more often affected with hypertrichosis than normal women (*Smith, 1944, Lohse & Bjarnhjedinnsson, 1945*), and that the adrenal cortex is of importance for hypertrichosis in women, have prompted me to study the 17-ketosteroid excretion in 64 women, comprising both schizophrenics and mentally normal (*Leth Pedersen, 1947*). Within both groups those with hypertrichosis as well as those with normal hairiness were examined. I thereby found a tendency to increased 17-ketosteroid excretion (and therefore probably to increased 17-ketosteroid production) in the hypertrichotic women, but independent of whether they suffered from schizophrenia or were mentally normal. Furthermore, there was found no increase in the 17-ketosteroid excretion in schizophrenic women with normal hairiness. Thus, the 17-ketosteroid production as such is hardly likely to play any part in the pathogenesis of schizophrenia. This does not preclude, however, that other functions of the adrenal cortex may be of pathogenetic importance.

Formerly *lesions of the female sexual glands* were supposed to be associated with specific psychosis. Menopausal psychoses, for instance, have been described, in particular the »involution melancholia« and paranoid mental diseases. However, it seems now to have been proved that specific menopausal psychoses do not exist. At the menopause there is found an increased psychic vulnerability and consequently increased morbidity with regard to the commonly known psychoses; and the menopause may to some extent mark these psychoses, particularly in the forms of anxiety or sexual colouring (*Dickmeiss, 1940*). The same is the case with regard to female castrates. Here, too, troubling mental symptoms are, as already mentioned, of frequent occurrence, but there is found no specific castration psychosis. Similar conditions assert themselves during the puerperium. Thus, there are hardly found specific puerperal or lactational psychoses, but during these periods there is increased morbidity with regard to the commonly known psychoses, presumably due to hormonal disturbances, possibly in connection with toxic influences.

It has been demonstrated both clinically and experimentally that the hypothalamus is of particular importance for the emotional life. Hence the investigations made within recent years into the aetiology and pathogenesis of the manic depressive psychoses have been concentrated particularly on the hypothalamus. However, we have not yet been able to prove that the manic depressive psychosis is released from a lesion or a dysfunction of the hypothalamus. An interesting observation is that of a relationship between thyroid lesions and manic depressive psychosis, which is suggestive of a common (probably hypothalamic) cause of the two diseases (*Ostenfeld, 1944 and 1947*).

Another point of importance for the study of the hypothalamus and its significance within psychiatry is that of the pathogenesis of *hysteria*. I have mentioned that in patients with pituitary-hypothalamic insufficiency I have found mental changes manifesting themselves particularly as increased irritability, emotional instability, and egocentricity, symptoms

which, when occurring jointly make these patients seem »hysterical«. I have observed similar mental symptoms, but coming on in fits, in other patients, who had psychomotor attacks as well (*Leth Pedersen*, 1948). These attacks were diagnosed as hysterical in neuropsychiatric departments; but encephalography revealed here cerebral atrophy of the ventricular type. In all these cases I find it reasonable to presume that the mental symptoms were due to lesion of the vegetative (or hormonal?) centres of the hypothalamus.

It is a well-known fact that hysterical symptoms may occur after organic cerebral lesions (among others encephalitis and cranial traumas). Moreover, it does not seem unlikely that the function of the vegetative centres in the diencephalon may be damaged with development of hysterical symptoms or other neuroses, as suggested among others by *von Monakow* in his so-called plexus-theory. *Von Monakow* states here that the bloodliquor barrier may fail because of functional influences, such as strong emotions and the like, by which the central nervous system is exposed to toxic influences, against which it is commonly protected. Even though the release of the hysterical reaction is mentally conditioned, there can hardly be any doubt that there is an underlying somatic predisposition, presumably a hypersensitiveness of the hypothalamic centres (*Brun*, 1946). This somatic predisposition is probably acquired in the majority of the cases.

SUMMARY

A brief survey is given of the occurrence of mental symptoms in association with endocrine lesions. The possibility is suggested of an endocrine aetiology of certain mental diseases, notably those with emotional instability and hysterical symptoms.

The author gives an account of own studies concerning patients with lesions in or about the hypothalamus and women castrated by operation. Rather characteristic mental symptoms have been found in both groups of patients, particularly with-

in the field of emotion, with symptoms of emotional instability and increased irritability, but not identical in the two groups. Thus the patients with hypothalamic lesion are characterized besides by a marked egocentricity, they being self-centred and hard to please, inert and despairing, whereas the female castrates by no means are self-centred, despairing or inert, but restless and rather minimizing their symptoms.

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THE URINARY EXCRETION OF
17-KETOSTEROIDS IN WOMEN DETERMINED
BY THE ZIMMERMANN REACTION*)

BY

MIRJAM FURUHJELM

A number of methods of determining the content of 17-ketosteroids in the urine are in use at present. For some years I have been using the *Zimmermann* reaction somewhat modified (*Furuhjelm*, 1940). This method is very simple and is well suited for routine examinations. A large series of healthy women have been investigated, which seems to be worth publishing, due to the fact, that continual determinations were made during the whole ovarian cycle. A certain insight into the question whether there are cyclic variations in the excretion of 17-ketosteroids in women have therefore been gained.

Most workers report that the excretion of 17-ketosteroids does not show cyclic variations. *Hülsmann* (1946) considers that it varies parallel with the excretion of oestrogenic substances and gonadotrophin. *Pincus* (1943) showed that the excretion decreases and is more constant during the sleep. The

*) This investigation has been carried out with the support of *Stiftelsen Therese och Johan Anderssons Minne*, Stockholm, for which I beg to offer my sincere thanks.

levels for the urinary excretion of 17-ketosteroids in women in the fertile age varies from 5 to 15 mg. per 24 hours (*Fraser, Forbes, Albright, Sulkowitch & Reifenstein, 1941*), 2 to 15 mg. (*Callow, Callow & Emmens, 1938*), 3.8—16.9 mg. (*Forbes, Donaldsson, Reifenstein & Albright, 1947*), 9 mg. (*Salter, Cohen & Sappington, 1946*) and 5 to 18 mg. (*Pincus, 1943*).

In gynecology the determination of the urinary 17-ketosteroids is of greatest importance in cases of masculinization, virilism and hirsutism. The presence of these symptoms compels us to differentiate between Cushing's syndrome, ovarian tumor and adrenogenital syndrome. A constitutional abnormal growth of hair not caused by any of these conditions is also frequently encountered. In those cases the origin of this symptom is obscure: exploratory laparotomy reveals no masculinizing ovarian tumor or gross adrenal lesion.

An increase of the urinary output of 17-ketosteroids is found primarily in carcinoma of the adrenal cortex and in hyperplasia of the adrenal cortex. Large quantities of 17-ketosteroids are often found in these cases (*Dobriner, 1942, Talbot & Butler, 1942, and others*). Virilism is accompanied by varying levels, usually within the normal range and only in the presence of pronounced symptoms of virility do they show an increase. In Cushing's syndrome one finds both normal and increased levels (*Hamblen, Cuyler & Baptist, 1941, Talbot & Butler, 1942, and others*).

I have determined the content of 17-ketosteroids in 29 women with more or less pronounced virile traits and in 6 patients suffering from Cushing's syndrome.

MATERIAL AND TECHNIQUE

1. *Healthy women.*

The 17-ketosteroid excretion in the urine was determined in 19 healthy women. The age of the subjects varied between 20 and 40 years (7 subjects: 21 to 25 years; 5: 26 to 30 years; 5: 31 to 35 years; 2: 36 to 40 years). Eighteen of the women were examined for one ovarian cycle, during which they sup-

plied us with their total urinary output. The nineteenth was studied in the same way for four consecutive cycles. Each determination of 17-ketosteroids was made on the urinary output for 48 hours, i. e. 14 determinations in a 28 days cycle,

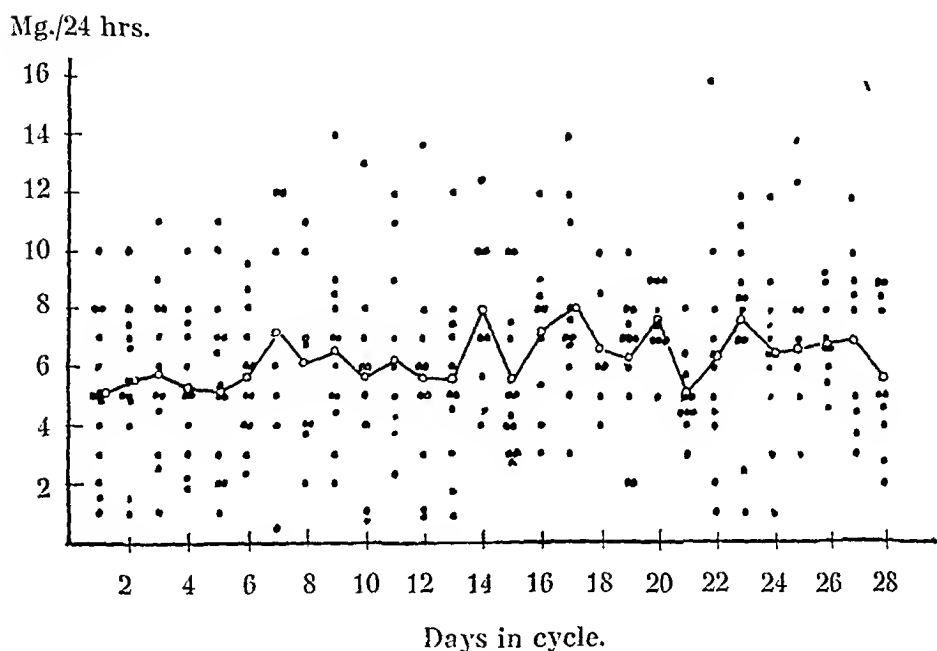


Fig. 1.

The urinary excretion of 17-ketosteroids in 19 healthy women during one menstrual cycle.

15 in a 30 days cycle, etc. The levels are given in milligrams per 24 hours.

The excretion of 17-ketosteroids varies from day to day. At its lowest it is about 1 mg. and at its highest 16 mg. (Fig. 1).

Most of the levels (170 of 297) are between 4 and 8 mg. per 24 hours. The excretion shows no definite cyclic variation corresponding to the ovarian cycle. However, a tendency toward a decrease in excretion during menstruation and to an increase during the secretory phase can be detected.

Table 1.

Case no.	Age years	Para	17-ketosteroids in mg./24 hrs.	Notes
1.	18	0	4, 4.4, 6.5, 12.0, 2.4, 2.6, 2.0	Operated on for corpus luteum cyst. Ovaries otherwise normal. Right adrenal gland felt to be slightly enlarged and was removed. It showed no definite histologic changes.
2.	22	0	5.1	Exploratory laparotomy: Ovaries normal.
3.	26	1	6.5	
4.	26	0	7.5	Exploratory laparotomy: Ovaries showed cystic degeneration. Palpation revealed the adrenal glands to be of normal size.
5.	27	0	12.5	Very obese.
6.	27	0	6.2	
7.	30	1	4.1	
8.	30	0	4.8	Exploratory laparotomy: Ovaries normal. The adrenal glands seemed normal upon palpation.
9.	32	0	16.0	
10.	32	0	6.5	
11.	32	0	1.9	Exploratory laparotomy: Ovaries normal.
12.	35	2	3.5, 5.1	Exploratory laparotomy: Ovaries normal. The adrenal glands seemed normal upon palpation.
13.	36	1	2.8	
14.	37	6	7.4, 7.4	Exploratory laparotomy: Ovaries normal. The adrenal glands seemed normal on palpation.
15.	37	0	5.2	

Urinary excretion of 17-ketosteroids in 15 women with regular menstruations but masculine hair growth,

Table 2.

Case no.	Age years	Para	17-ketosteroids in mg. /24 hrs.	Notes
16.	18	0	10.0	Exploratory laparotomy: Ovaries normal. The adrenal glands seemed normal on palpation.
17.	21	0	15.0 before and 6.6 2 months after	Ovaries normal. On exploratory laparotomy left adrenal gland was felt to be enlarged, for which reason it was electrocoagulated at a later operation.
18.	20	0	9.0	Exploratory laparotomy: Ovaries normal. Palpation revealed adrenal glands to be normal. Normal parturition 2 years later.
19.	22	0	8.7	
20.	26	0	13.6	Exploratory laparotomy: Ovaries normal. The adrenal glands seemed normal on palpation.
21.	26	1	9.1	
22.	32	0	5.1	Adiposity.
23.	38	0	7.6	Exploratory laparotomy: Follicular cysts in both ovaries. Palpation revealed adrenal glands to be normal.
24.	38	0	5.0	
25.	20	0	13.0	Amenorrhea.
26.	23	0	10.0	Amenorrhea. Exploratory laparotomy: Follicular cysts in both ovaries. Palpation revealed adrenal glands to be normal.

Urinary excretion of 17-ketosteroids in 11 women with pronounced virile hair growth and hypo-oligomenorrhea or amenorrhea.

2. Patients with virile symptoms.

This series includes 29 cases of virilism. It is divided into 3 groups.

a) the first group contains 15 patients with regular menstruations but masculine hair growth, which was so pronounced that it led to the patients being hospitalized. The age of the patients varied between 18 and 37 years. The 17-ketosteroid content of the urine was determined once only in most of the cases, and the levels were within the normal range of variations. (Table 1).

The ovaries were split and a specimen was taken for microscopic examination in all the cases submitted to exploratory laparotomy.

b) The second group includes 11 patients with pronounced virile growth of hair and hypo-oligomenorrhea in nine cases and amenorrhea for more than one year in two cases. The age

Table 3.

Case no.	Age years	Para	17-ketosteroids in mg./24 hrs.	Notes
27.			22.4, 6.9	Exploratory laparotomy: Follicular cysts in both ovaries. Palpation revealed adrenal glands to be normal.
28.	20	0	19.2	Exploratory laparotomy: Ovaries normal. Excision of left adrenal gland, but no definite pathological changes demonstrable histologically.
29.	35	0	59.0, 57.0, 55.0, 45.0, 36.0	Biopsy from left adrenal gland normal. Right adrenal gland enlarged. Biopsy showed hyperplasia. Exploratory laparotomy: Ovaries normal.

Urinary excretion of 17-ketosteroids in 3 women with pronounced masculinization and amenorrhea.

Table 4.

Case no.	Age years	Para	17-ketosteroids in mg./24 hrs.	Notes
30.	25	0	13.3, 6.6	
31.	30	0	4.0	
32.	25	0	35.0, 15.0, 12.0	
33.	36	0	29.0, 49.0	
34.	40	0	20.3	
35.	50	0	10.7	Left-sided renal incision. Left adrenal gland of normal size. Biopsy from left adrenal gland showed fairly profuse accumulation of ponceau-fuchsinophil granules in the cortical cells, mainly in the internal layers. X-rays following injection of air around the right kidney revealed no enlargement of the right adrenal gland.

Urinary excretion of 17-ketosteroids in 6 women with Cushing's syndrome.

of the patients varied between 18 and 38 years. No definitely pathological levels could be shown in this group either. (Table 2).

As in the previous group biopsy specimens were taken in the cases submitted to exploratory laparotomy from both ovaries.

c) The third group includes three patients with amenorrhea and pronounced virile symptoms: masculine hair growth, deep voice, masculine pelvis and hypertrophy of the clitoris. All three showed an unusually high level of 17-ketosteroids in the urine. These cases are collected in Table 3.

3. *Patients with Cushing's syndrome.*

The urinary excretion of 17-ketosteroids was determined in six cases diagnosed clinically as Cushing's syndrome. Three of them showed an increase in excretion, while the levels in the remainder were normal. (Table 4).

DISCUSSION

The levels for the urinary excretion of 17-ketosteroids in women in the fertile age found in this investigation correspond well with those usually reported in the literature. The question whether the excretion of 17-ketosteroids exhibits regular variations during the ovarian cycle has been discussed, the majority of workers being of the opinion that it does not. In my publication in 1940 I stated that there was a certain tendency toward parallelism between the excretion of oestrogenic substances and of 17-ketosteroids in normal women. However, unlike the oestrogenic excretion, the excretion of 17-ketosteroids showed no regular, pronounced peak in the middle of the cycle, nor did it reach exceptionally low levels during menstruation. The curve representing the normal cases (Fig. 1) seems to show a tendency toward higher levels during the secretory phase. The curve rose on the 14th day, remaining at the same level until the next menstruation, when it dropped again. It seems reasonable that there may be a certain cyclic variation in the excretion of 17-ketosteroids in women. In castrated young experimental animals the adrenals affect the genital organs in a way reminiscent of the ovaries, and cyclic variations analogous to those in the non-castrated can be produced in the genital organs of the castrated animal. Furthermore, repeated, regular bleeding that resembles menstruation may be observed in castrated women who have received continuous medication with large doses of oestrogenic substances (Parkes, 1945).

Among the women with virilism, the highest 17-ketosteroid levels were found in those with most pronounced virile symptoms. Like most other workers I found the levels in the milder cases to be within the range of normal variations, irrespective of whether menstruation was normal. A differentiation of the various 17-ketosteroids in these cases might yield interesting information. In contrast to our results *Hamblen, Cuyler & Baptist* (1941) reported that they found increased 17-ketosteroid levels in nearly all their cases both of moderate and of pronounced virilism.

Three of the women with Cushing's syndrome showed increased levels in the 17-ketosteroid excretion. The other had an output within the normal limits.

SUMMARY

The *Zimmermann* reaction was found to give approximately the same results as other methods of determining the 17-ketosteroid excretion in the urine reported in the literature. In a series of 19 healthy women studied for at least one ovarian cycle, the urinary output of 17-ketosteroids was found to vary between 1 and 16 mg. per 24 hours. Most of the levels were between 4 and 8 mg. (170 of 279 determinations). There seemed to be a tendency toward higher levels during the secretory phase. Of 29 cases of virilism, three with strongly pronounced virile symptoms showed an increased urinary excretion of 17-ketosteroids. Three of the six cases of Cushing's syndrome showed an increased 17-ketosteroid level.

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THE ANTAGONISTIC ACTION OF METHYLTHIOURACIL ON THE INHIBITORY EFFECT OF THYROXIN ON SERUM LIPASE

BY

GEORGE KLEIN

Bach, Lovas & Neufeld (1932) have demonstrated that the level of serum lipase in rabbits is diminished after subcutaneous injection of 0.25—1.0 mg. thyroxin. According to these authors the value of serum lipase, expressed as the velocity constant of tributyrin hydrolysis, is lowered by $\frac{1}{3}$ 24 hours after the injection of 0.5 mg. thyroxin, and to $\frac{1}{2}$ of the original value after 48 hours; the original level is reached after 3—5 days. This effect of thyroxin was thought to be an indirect one as no inhibition of lipase could be produced *in vitro*.

These results were confirmed by *Fiessinger, Albeaux-Fernet & Gajdos* (1933), *Hoffmann* (1934), and *Bauer & Feil* (1935). *Hoffmann* and *Bauer & Feil* investigated the possibilities of inhibiting this effect of thyroxin. They found that the thyroxigen breakdown of serum lipase could be compensated by a number of lipid substances. The insulin-free pancreatic extract of *Baló, Bach, Lovas & Neufeld* (1932), Retardin, is also able to compensate this effect and protects the animals from lethal thyroxin intoxication at the same time. According to *Baló* and co-workers every substance which is capable of inhibiting the effect of thyroxin on serum lipase is able to pro-

tect rabbits from thyroxin-death. Out of these reasons it was thought worth while to study the antagonism between thyroxin and serum lipase from another angle.

The aim of the present work is to investigate the possibility of inhibiting the lowering effect of thyroxin on serum lipase by compounds known to inhibit thyroxin formation, viz. methylthiouracil.

EXPERIMENTAL

Methods.

Determination of serum lipase. The stalagmometric method of *Rona & Michaelis* (1911) was used.

In every determination there were mixed 50 ml. of saturated tributyrin solution (10 drops of tributyrin mixed with 1 liter aqua dest., shaken for two hours, filtered), 3 ml. phosphate-buffer (see. Sørensen, 1 p. 1/3 n sol. prim. phosph. is mixed up with 7 p. 1/3 n sol. sec. phosph., H^+ conc. (=) $0.35 \cdot 10^{-7}$) and 0.25 ml. rabbit serum. The hydrolysis took place in water bath with a temperature of 22° C. The stalagmometric determination was made with a pipette as described by *Michaelis* (1926). As a rule the number of drops was determined every 10 minutes. The velocity of tributyrin hydrolysis was expressed by the constant (k) of the monomolecular reaction, according to *Rona & Ebsen* (1912), calculated by the following formula:

$$k = \frac{1}{t} \log \frac{a}{a-x}$$

where k = velocity constant of the monomolecular reaction, t = time in minutes, a = concentration of tributyrin at time 0, $a - x$ = concentration of tributyrin at time t .

The final k -values used were means of 5 determinations of k .

The error of a single determination was established by calculating the standard deviation (σ) of these five determinations. In all 36 series, each computing 5 determinations, on 8 animals in all, were used for calculation.

The standard deviation was found to be $1.781 : 10^{-3}$ on an average (expressed in k-value), and varying between $0.277 \cdot 10^{-3}$ and $5.550 \cdot 10^{-3}$. The variation corresponds to 8.14 per cent of the mean.

The error of the method, as the final value was the mean of 5 determinations, was thus: $\frac{1}{\sqrt{5}} \cdot 8.14 \text{ per cent} = 3.64 \text{ per cent.}$

Experimental Design.

Rabbits, weighing about 2000 gm., were used for the experiments. The diet of the animals was constant and their weight did not change during the experiment.

In preliminary orientating experiments the effect of treatment with methylthiouracil alone was investigated on the level of serum lipase of normal animals. No effect was obtained with small doses, from larger doses definite inhibition could be seen. In the following experiment the largest dose of methylthiouracil was used which did not inhibit serum lipase if given alone. This dose was found to be about 0.025 gm./kg.

The experiments were made as follows:

The tributyrin hydrolysing capacity of the serum of untreated animals was determined twice at least before treatment. After this 0.25 mg./kg. thyroxin (La Roche) was injected subcutaneously to the *first* series of rabbits. Serum lipase determinations were made after 48 hours and again after 10—14 days. In the last mentioned determination the serum lipase level was always found to be at the original height. Having found this the second treatment was made: 0.025 gm./kg. 4-methyl-2-thiouracil was given through stomach tube and that was followed after 4 hours by the administration of 0.25 mg./kg. thyroxin subcutaneously. Lipase determinations were made after 48 hours and after 10—14 days. The *second* series of rabbits was treated in the same way except that the experiment was started with the described combined administration of thyroxin and methylthiouracil and finished with the administration of thyroxin alone.

RESULTS

1. *Variability of normal values.* The k-values are rather constant under normal conditions, but may show variations from time to time. The variability was established by analysis of variance (Fisher, 1936, and Bonnier & Tedin, 1940) by

Table 4.

Example for the statistical analysis of variance in one animal. A: Variation of normal values from time to time, B: Variation between normal values and values obtained after thyroxin treatment, C: Variation between normal values and values obtained after the combined treatment with thyroxin + methylthiouracil.

P: Probability that the variation is due to chance.

Example for the statistical analysis of variance.

Rabbit no. VI.

Variation	Degrees of freedom	Sum of squares	Mean square	Standard deviation	Standard deviation in per cent of mean
<i>A. Normal values.</i>					
Between the groups	2	0,000.005.6	0,000.002.80	0,001.673	5,80
Within the groups	12	0,000.044.9	0,000.003.74	0,001.934	6,70
Total	14	0,000.050.5	0,000.003.61	0,001.900	6,65

Variance ratio = 1,34 (df = 12/2) $P > 0,2$

B. Effect of thyroxin.

Between treated and normal	1	0,000.887.19	0,000.887.19	0,029.782	118,98
Within the groups	18	0,000.051.23	0,000.002.85	0,001.688	6,74
Total	19	0,000.938.42	0,000.049.39	0,007.028	28,08

Variance ratio = 311,29 (df = 1/18) $P < 0,001$

C. Effect of thyroxin + methylthiouracil.

Between treated and normal	1	0,000.002.23	0,000.002.23	0,001.493	5,12
Within the groups	18	0,000.064.13	0,000.003.56	0,001.887	6,47
Total	19	0,000.066.36	0,000.003.49	0,001.837	6,30

Variance ratio = 1,60 (df = 18/1) $P > 0,2$

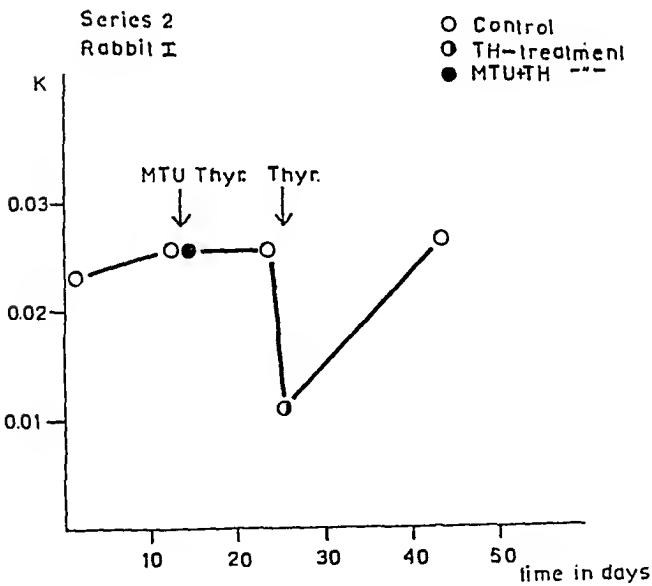
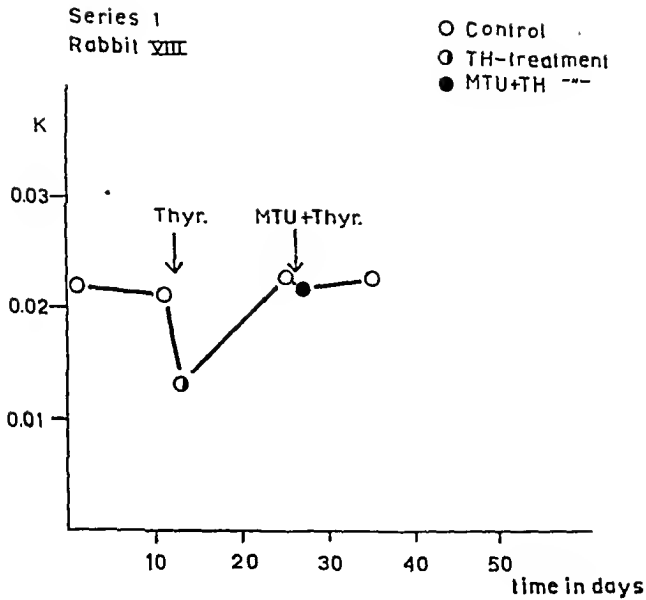


Fig. 1 and 2.

Typical examples of the changes obtained after treatment with thyroxin alone and after combined treatment with thyroxin + methylthiouracil in the same animal, k = velocity constant of tributyrin hydrolysis.

Table 2.
Survey of the changes obtained after thyroxin treatment and after
the combined treatment with thyroxin + methylthiouracil.

the same

Series 1.

Nr. of rabbit		NORMAL		THYROXIN 48h after injection			THYROXIN + METHYLTHIOURACIL 48h after administration				
		Nr. of de-term.	k value mean	Nr. of de-term.	k	Change in per cent from normal	P *	Nr. of de-term.	k	Change in per cent from normal	P *
II.*	20		0,0139	5	0,0080	-42 %	< 0,001	5	0,0149	+ 7 %	> 0,2
VI.	15		0,0288	5	0,0137	-52 %	< 0,001	5	0,0301	+ 5 %	> 0,2
VII.	15		0,0140	5	0,0042	-70 %	< 0,001	5	0,0126	-10 %	0,2 > P > 0,05
VIII.	15		0,0228	5	0,0135	-41 %	< 0,001	5	0,0225	-1 %	0,05 > P > 0,01
						mean = -51 %		mean = + 0,25 %			

Series 2.

I.	20	0,0252	5	0,0113	-55 %	< 0,001	5	0,0256	+ 2 %	> 0,2	
III.	20	0,0138	5	0,0074	-46 %	< 0,001	5	0,0121	-12 %	> 0,2	
IV.	14	0,0171	5	0,0124	-38 %	0,01 > P > 0,001	5	0,0151	-12 %	> 0,2	
V.	20	0,0349	5	0,0156	-55 %	< 0,001	5	0,0268	-23 %	0,01 > P > 0,001	
						mean = -48 %		mean = -11 %			

* P - probability that the variation is due to chance

* P — probability that the variation is due to chance

comparing the variation *within* a determination with the variation *between* different determinations, made under normal conditions. (See Table 1/A). In 6 out of 8 rabbits there was no difference between the variation *within* and the variation *between*, which means that the variation of k-value from time to time (about 6 weeks in all) did not exceed the experimental error. In 2 rabbits the k-values were increased 10 days after thyroxin treatment, but there was no difference between the two values before treatment.

The variation from time to time, expressed as the standard deviation (σ) in k-value, calculated from the control determinations — at least four determinations from each animal — varied between $1.063 \cdot 10^{-3}$ and $6.550 \cdot 10^{-3}$, with a mean value $3.163 \cdot 10^{-3}$, which corresponds to 1.14 per cent of the mean.

The k-values are thus rather constant in one animal, but are very different in different animals. These findings confirm the statement of *Baló, Bach, Lovas & Neufeld* (1932) as to k-values in normal animals.

2. *Effect of thyroxin-treatment.* 48 hours after the injection of thyroxin alone the serum lipase values fell significantly whether thyroxin was injected first (series I) or two weeks after a previous administration of methylthiouracil + thyroxin (series II) no difference being found between the two series. The decrease was established by analysis of variance (see example in Table 1/B). The decrease was — 51 % in the first, — 48 % in the second series. Typical experiments are given in Figs. 1 and 2, a survey is given in Table 2.

3. *The effect of combined treatment with thyroxin and methylthiouracil.* 48 hours after the combined administration of methylthiouracil and thyroxin no statistical difference in serum lipase could be seen in 6 of 8 animals, established by analysis of variance (example Table 1/C). Some typical cases are illustrated in Figs. 1 and 2, a survey is given in Table 2.

In one animal of the first series (VIII) there was a statistically probable decrease, in one of the second series the decrease was highly probable (V). However, in both cases the lowering is much less than the diminution obtained after treatment with thyroxin alone in the same animal. (Table 2).

On this basis it can be concluded that the decrease of serum lipase caused by one single injection of thyroxin can be inhibited by methylthiouracil.

DISCUSSION

As the normal thyroid gland has a thyroxin supply, which is able to produce a normal basal metabolism in the first 1—2 months of thiourea treatment (*Astwood*, 1943), the effect of methylthiouracil here presented, inhibiting the effect of thyroxin on serum lipase, cannot be explained by a direct action causing a decrease of hormone streaming from the thyroid into the circulation as the effect appears within 48 hours, but must be supposed to be a peripheral one.

The possibility of a peripheral effect of thiouracil on other functions is recently discussed by several authors (*Abelin*, 1945, 1947, *Gasche*, 1946, and *Poupa*, 1946). A number of authors have found effects of thiourea compounds upon different enzymes (*Bernheim & Bernheim*, 1942, *Paschkis*, *Cantarow & Tillson*, 1945, 1947, *Paschkis*, *Cantarow*, *Tillson & Rakoff*, 1945, *Lerner & Chaikoff*, 1945, *Kenneth & Erway*, 1946, *Tipton & Nixon*, 1946, *Glock*, 1946, and *Fontaine & Leloup*, 1946, 1947).

Thus evidence is gathering as to there being some peripheral effects of thiourea compounds, which is supported by the findings here presented: the antagonism demonstrated between thyroxin and thiouracil as to influence on serum lipase.

I wish to acknowledge my great indebtedness to Docent *Leonard Goldberg*, Karolinska Institute, *Doctor Rolf Luft*, *Serafimerlasarettet*, and *Professor Joseph Baló*, University of Budapest for the interest they have shown in this investigation and their valuable advice and criticism.

SUMMARY

The administration of 0.025 gm./kg. methylthiouracil to rabbits by stomachtubing inhibited the serum lipase reducing effect of 0.25 mg./kg. thyroxin given subcutaneously. After the injection of thyroxin a statistically significant decrease of serum lipase was obtained in 8 animals. With the administration of methylthiouracil this effect could be completely inhibited in 6 of 8 animals, where no statistical difference could be obtained. In one animal there was a statistically probable, in another animal a statistically highly probable decrease after this treatment, but the decreases were much less than the lowering obtained after thyroxin treatment in the same animal.

No difference could be seen whether thyroxin was injected first or two weeks after a previous administration of thyroxin + methylthiouracil.

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ON THE ACCURACY OF METHODS FOR DETERMINING THE POTENCY OF GROWTH HORMONE PREPARATIONS

BY

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From the paper »A Study on Growth Hormone of Anterior Pituitary Lobe«, recently published by *Fonss-Bech* (1947), it seems possible to gain some information regarding the accuracy of various methods for determining the potency of growth hormone preparations. It is claimed that two methods are of special interest, viz, the dwarf mice method and the »plateaued« female rats method. Both methods are described in detail in the paper cited.

According to standard methods for the evaluation of biological assays, the accuracy of a method is measured by the ratio, λ^2 , of the variance to the squared slope of the log dose-response line, the estimate of which ratio is $\lambda^2 = \frac{s^2}{b^2}$ (*Bliss & Cattell*, 1943). From the data presented in the paper by *Fonss-Bech* (1947), table 13, col. 2, p. 74 and table 28, p. 104 it is easily calculated that $\lambda^2 = 0.449$ for the dwarf mice and $\lambda^2 = 0.051$ for the plateaued female rats. It follows, that if the methods were to show the same degree of accuracy, then the number of dwarf mice should exceed that of the plateaued female rats $\frac{0.449}{0.051} = 8$ times.

The plateaued female rats method, therefore, seems to be much superior to the dwarf mice method. Furthermore, it

seems to be as satisfactory as any other method described in the literature. From the data of *Bülbring* (1938) and *Marx, Simpson & Evans* (1942) on the hypophysectomized rats method, *Bliss & Cattell* have calculated I^2 to be 0.125 and 0.070 resp., both of which exceed $I^2 = 0.051$ calculated from *Fonss-Beck's* data on the plateaued female rats method. This author has also investigated the hypophysectomized rats method and the »increase in tail-length« method. From the data on these methods I^2 is calculated to be 0.162*) and 0.283**), respectively.

As to the dwarf mice method and the plateaued female rats method the author himself has computed the I^2 (assisted by *Borge Andersen*, M. Sc.). He finds for the dwarf mice $I^2 = 0.0550$ and for the female rats $I^2 = 0.0702$ and consequently that about $\frac{0.0702}{0.0550} = 1.3$ times as many rats are required as dwarf mice. His figure for the female rats is, however, simply due to an arithmetic error. It may be attributed to the sum of the figures of the last column (p. 104 l. c.), — 81.3000 instead of 77.1100; hence $b = 18.07$ and $s^2 = 16.74$ (instead of 17.14 and 20.62 resp.); thus $I^2 = 16.74 : 18.07^2 = 0.051$ instead of 0.0702.***)

Stranger yet is the treatment of the data on the dwarf mice. 40 mice were applied in the case of each dose but only 10 of them were submitted to the statistical analysis. Thus 75 per cent of the experimental work has not been utilized in the evaluation of the method. The 10 mice per dose were admittedly picked at random (p. 80 l. c.), but the effect has been in favour of the dwarf mice method. The diagrams below are rather

*) Deduced from table 35, p. 120, l. c.

**) Deduced from table 38, p. 128, l. c. after correction for an arithmetic error in the last column of the table — 13.3500 instead of 12.3500. Omitting the highest dose, however, I^2 for the tail-length method becomes 0.107, a figure which indicates a tolerable precision.

***) Readers of *Fonss-Beck's* paper should also be reminded of an arithmetic error in the last column of table 34, p. 118 l. c. — 57.3000 instead of 53.3000. Like the other two, this error is disfavoring the method involved.

instructive: Diagram 1 shows the weight increases of the mice drawn »at random« and the reaction line calculated (omitting the highest dose) (cf. table 15, p. 82 l. c.). Table 13 (p. 74 l. c.) presents, however, a summary of all data, giving the mean value for groups of 10 animals. From this, diagram 2 has been

*Reactions of dwarf mice treated for 2 weeks
with hormone extract.*

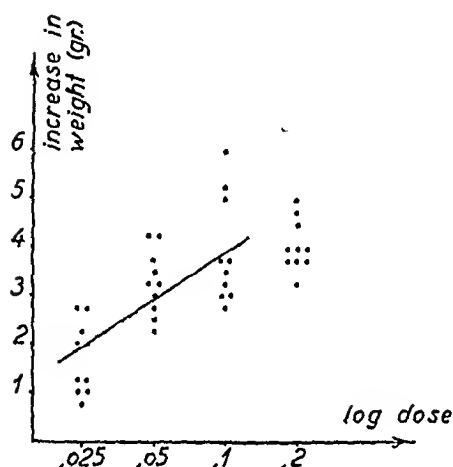


Diagram 1.

The points indicate figures from table 15 (p. 82 l. c.), each figure being the weight increase for a »random« dwarf mouse. (The reaction line is that of p. 89, l. c.).

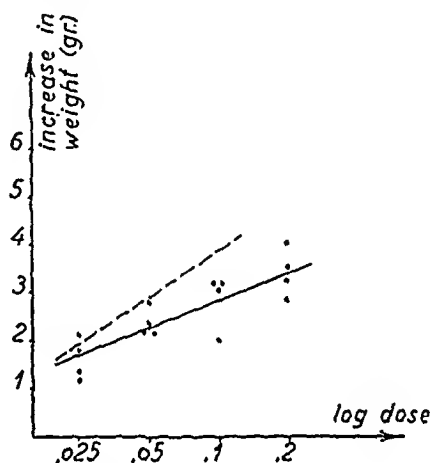


Diagram 2.

The points indicate figures from table 13 (p. 74 l. c.), each figure being the average weight increase for 10 mice. All mice are represented here. (The reaction line is that of p. 78 l. c.).

constructed, the line drawn in full being the estimated reaction line (now with no plausible reason for omitting the highest dose). For illustrative purposes the reaction line of diagram 1 has also been indicated. Obviously, the slope turned out to be much larger for the animals selected »at random« than for the total material.

The »random procedure« has led to a selection of the animals with higher weight increases on the doses 0.05 ml. and 0.10 ml., thus increasing the slope and reducing the mean

square. Consequently, an I^2 computed from the »random mice« is strongly biased in favour of the dwarf mice method. This seems to be the main reason for *Fønss-Bech's* obvious over-estimation of the accuracy of the dwarf mice method. As matters stand, the dwarf mice method must be regarded as being of doubtful value in spite of its apparent specificity and adequacy.

Before the plateaued female rats method can be recommended, it should be remembered that according to current views on biological standardization a relevant determination of the potency of a preparation requires a comparison with a standard. In order to justify such a measurement it is a necessary condition that the log dose-response curves for the preparation and the standard are parallel. In the present investigation only one preparation has been used. Consequently no potency determination in the usual sense has been carried out, nor has it been possible to test the basic hypothesis on parallelism.

There is some evidence, however, of parallelism between log dose-response curves for different preparations of the growth hormone. In her paper on the reaction curve of the hypophysectomized rats method *Bjölbring* (1938) finds that the curves for various extracts of the growth hormone may be considered parallel. *Marx, Simpson & Evans* (1942) state that curves representing the relationship between dose and response for different growth hormone preparations have a constant slope. They investigated the plateaued female rats method and the hypophysectomized rats method. In their paper seasonal variations in responsiveness of the rats are discussed and the importance of a standard reference substance is stressed.

A comparison of the two methods examined led the authors to the result that the accuracy was approximately the same for both types of test animal. It appeared to be even somewhat higher for normal rats. *Marx, Simpson & Evans* (1942) were able to perform assays with hypophysectomized rats, applying doses of about one-tenth the doses needed for assays with normal plateaued rats. So the hypophysectomized rats method

should be used, when testing more valuable preparations. In this connection it is noticed that *Fonss-Bech* uses equal amounts of substance for both methods of assay.

After all, it seems justified at present to adopt the plateaued female rats method as the most accurate method for determining the potency of growth hormone preparations. Considering also its relative simplicity this method seems to deserve recognition.

SUMMARY

The accuracy of various methods for determining the potency of growth hormone preparations is discussed. The plateaued female rats method is recommended because of its relatively high accuracy and simplicity. It seems possible to save some amount of substance when using the hypophysectomized rats method. The accuracy of the dwarf mice method is rather low. It is shown that *Fonss-Bech* (1947) grossly overestimated the precision of this method. The »increase in tail-length« method may be fairly precise.

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OESTROGENIC AND GONADOTROPHIC
SUBSTANCES IN THE URINE FROM A WOMAN
WITH NORMAL MENSTRUAL CYCLES AND
NORMAL PREGNANCIES
SEX HORMONE ANALYSES III.*)

BY

G. PEDERSEN-BJERGAARD and K. PEDERSEN-BJERGAARD

In a preceding report from this laboratory *Pedersen-Bjergaard & Tonnesen* (1948 a) have investigated the excretion of gonadotrophic hormone and oestrin with the urine in normal, non-pregnant women. A fairly large material was examined, chiefly with one or some few analyses of each of the hormones per individual. The present work deals with the hormonal excretion mainly of only one individual, a woman in good health. All the urine voided was collected with particular care without a single interruption throughout a period of two years and some years later through 10 months. Two normal pregnancies occurred during these periods.

MATERIAL AND TECHNIQUE

The woman (Mrs. YY) was 31 years old at the commencement of the experiment. The menarche occurred at the

*) »Sex Hormone Analyses II« appeared in *Acta med. Scandinav.*, Suppl. 213, 1948.

age of 14, the first birth at the age of 29 years. The length of the menstrual cycle has fairly constantly been 26 days (see fig. 1), the shortest cycle amounting to 23 days, the longest to 29 days. These figures do not include the last two periods recorded, which amounted to 42 and 30 days respectively, as they fall entirely outside the otherwise regular rhythm. The probable cause of this sudden protraction of the interval will be discussed later on in this work. The duration of flow was 4 days. A period of 2 years from the age of 31 to the age of 33 with normal menstrual cycle was followed by a pregnancy where with the technique employed a rise in the output of gonadotrophic hormone could be demonstrated from the 20th day of the cycle. The pregnancy and parturition, with delivery of twins, took a normal course. The parturition took place 246 days after the day when the gonadotrophin excretion began to rise. It was followed by a lactation amenorrhea that lasted 65 days, and then normal menstruation reappeared with a cyclic period of 35 days. The collection and analysis of the urine was continued for 7 months after the parturition. 4 years later, the urine of the same woman was examined again through a period of nearly 10 months including a normal pregnancy that lasted 256 days. The urine was examined for one month preceding this pregnancy. As in the first pregnancy, the rise in the output of gonadotrophic hormone could be demonstrated from the 20th day of the menstrual cycle. After delivery, the urine was analysed only through one week.

The urine was collected as 48-hour specimens. For the preparation of gonadotrophic and oestrogenic urine extracts and the determination of the hormonal contents of these, the same technique was employed as has been described by *Pedersen-Bjergaard & Tønnesen* (1948 a) with this exception: the urine from the periods of pregnancy was not subjected to extraction, but injected directly, in suitable dilution, into test animals. One R. U. of gonadotrophin means the amount that produces a tripling of the uterine weight in 26 days old rats. One M. U. of oestrin means the amount that produces vaginal cornification in 60—70 per cent of the adult spayed mice employed.

RESULTS

1) *Gonadotrophic and oestrogenic substances during normal menstrual cycles.*

The values recorded in Fig. 1 give the 24-hour output. It will be noticed that until July 1935 the analyses covered only the output of oestrin. Not until September 1935 was our method for assay of gonadotrophic hormone elaborated so far that it could be used continuously.

The lowest oestrin value recorded was 8 and the highest 360 M. U. per day. The excretion of oestrin is lowest during the menstrual period. In the intermenstrual period the excretion increases, though without following any constant course, not even two among the 24 cyclic periods showing the same output of oestrin. Twelve periods show an excretion with 2 maxima, the first appearing on an average about the 10th day of the cycle, the other appearing on an average on the 21st day. This is in keeping with the findings reported by several other investigators, *e. g.* Genell (1943). But, while Genell thinks that this excretion is normal for women with normal menstruation, Fig. 1 shows that 8 periods presented merely one maximum, on an average about the 13th day of the cycle. If the oestrin output may be taken as a measurement of the ovarian function, the latter will have to be looked upon as not particularly regular from one cycle to another. It is even possible, we think, that sometimes one follicular rupture may take place on the 13th day of the cycle, sometimes two follicular ruptures on the 10th and 21st days of the cycle respectively.

Calculating the average output of oestrin from the values recorded in Fig. 1, leaving out the last two cyclic periods of respectively 42 and 30 days, we have the results presented in Fig. 2.

It will be noticed that the average excretion of oestrin attains its first maximum on the 12th day of the cycle. Another less pronounced maximum is reached on the 21st day. The average excretion was within the range 44—157 M. U. of oestrin per day.

A particularly noteworthy feature is the extraordinarily high output of oestrin, which — as is evident from Fig. 1 — was demonstrated from March 4—10, 1936, corresponding to the 17th to 23rd days of the cycle. During this week the output



Fig. 2.

The average oestrin output in M. U. in the same individual (Mrs. YY) through a 2-year period. Menstruation was present during the first 4 days.

of oestrin amounted to about 500 M. U. per day, and during the following menstrual period it did not fall as usual to values below 50 M. U. per day, but during the menstruation and in the following week it remained at a level of 250 to 300 M. U. This made the cycle in question particularly prolonged, lasting 42 days as against the usual 26 days. The observation that such an ovarian hyperproduction of oestrin is able to prolong the intermenstrual period by about 60 % is in keeping with the results obtained by *Okamoto, Yamamoto,*

Yagi & Kosakae (1935) by administration of oestrogenic substances to normally menstruating women.

From Fig. 1 it is evident that the excretion of gonadotrophic hormone is even more irregular than that of oestrin, the lower

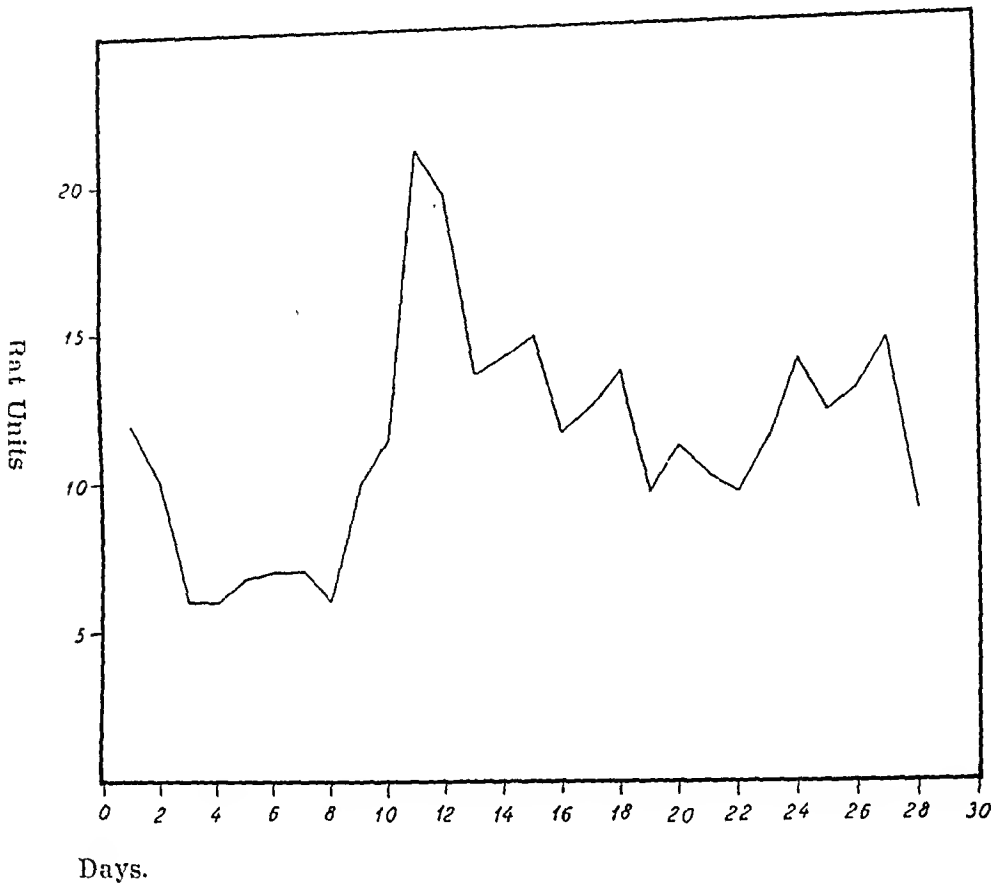


Fig. 3.

The average output of gonadotrophic hormone in R. U. in the same person (Mrs. YY) in 6 cyclic periods. Menstruation was present during the first 4 days.

and upper limits being 2 and 25 R. U. per day. The average gonadotrophic output calculated on the basis of the values obtained from 6/9—35 to 17/2—36, i. e., 6 cyclic periods, is recorded in Fig. 3. Here the average curve shows variations from 6 to 21 R. U. per day. It reaches its maximum on the 11th

day of the cycle, i. e., 1 day before the oestrin output becomes maximal (Fig. 2). *Levin* (1941) found the gonadotrophin output to reach its maximum on the 12th day in a woman with a cycle of 28 days, and on the 13th day in a woman with a cycle of 30—34 days. In both cases only one period was assayed for gonadotrophin.

No particular parallelism between oestrin and gonadotrophin excretion is evident from the findings recorded in Fig. 1. Still, the gonadotrophin output was strikingly low in the period when the oestrin output was extraordinarily high (March 1936). *D'Amour & Wood* (1941) studied the oestrogen and gonadotrophic hormone excretion in a normal woman during 18 consecutive menstrual cycles. More than one gonadotrophic hormone peak occurred in a number of cycles. Oestrogen excretion reached its maximum in 2 waves, usually preceding the gonadotrophic peaks.

2) *Gonadotrophic and oestrogenic substances at the onset of pregnancy.*

Several papers have been published on the early diagnosis of pregnancy based on increased excretion of gonadotrophic hormone with the urine. Thus, *Zondek* (1931) reported a positive *Aschheim-Zondek* reaction in two young girls several days before the first missing menstruation. In 1933 *Hamburger*, employing the *Aschheim-Zondek* reaction, was able in one case to demonstrate an increased output of gonadotrophin on the 26th day of the cycle, two days before the first missing menstruation. The exact time when the excretion of gonadotrophic hormone begins to increase, however, may be ascertained only by following this excretion in the same individual from day to day. By adopting this method, *Levin* (1941) has been able in one case to ascertain an increase in the output of gonadotrophin from the 24th day of the cycle. As is evident from table 1, we have been able to ascertain in the same individual the onset of 2 pregnancies, 5 years apart, on the basis of an increase in the gonadotrophin output from the

Table 4.

Oestrin and gonadotrophin output in the same person (Mrs. YY) measured at the beginning of the pregnancies in 1936 and 1941, respectively.

Days of the cycle	Output of oestrin (M. U./24 hrs.)		Output of gonadotrophin *) (R. U./24 hrs.)	
	1936	1941	1936	1941
12—13	42	17	7½	15
14—15	125	25	10	5
16—17	125	25	15	7½
18—19	167	25	7½	5
20—21	250	150	90	45
22—23	200	250	450	180
24—25	250	200	1800	450
26—27	350	300	1800	2100
28—29	300	250	4500	3000
30—32	250	350	18000	8500
33—34	250	250	18000	18000
35—36	350		18000	
37—38	350		45000	
39—40	350		90000	
41—43	400	250	90000	400000
44—46	300		180000	
47—49	400		180000	
50—52	400		500000	

20th day of the cycle, corresponding to the 16th day after the last day of menstruation.

It will be noticed that features characteristic of the onset of pregnancy are: 1) cessation of the cyclic variations in the oestrin output, which in the beginning of pregnancy keeps

*) As most of the analyses in this work were carried out before the establishment of the international chorionic gonadotrophin standard, only the direct rat units (R. U.) are recorded here, without conversion to international units. With the technique here employed 1 R. U. chorionic gonadotrophin corresponds to 1/5 I. U. The values for gonadotrophin excreted in non-pregnant women are due to hypophysial gonadotrophin, and they cannot be converted into I. U.

fairly constant at a level of about 200—400 M. U. per day; and 2) a greatly increasing gonadotrophin output, commencing on the 20th—21st day of the cycle, and continuing the increase during the first month of pregnancy. Thus, the increase in gonadotrophin excretion begins 8—9 days after the maximal oestrin output of the normal menstrual cycle is ascertained (Fig. 2), and it seems reasonable to assume that the excretion of chorionic gonadotrophin starts simultaneously with the implantation of the fertilized ovum in the uterine mucosa.

In this connection, it is rather interesting that in the mare the production of serum gonadotrophin commences at the same time as chorionic villi begin to develop on the surface of the fertilized ovum, but this production ceases at the juncture when the attachment of the ovum to the uterine mucosa takes place (*Glud, Pedersen-Bjergaard & Portman, 1933*). Demonstrable amounts of gonadotrophin were found in the serum of the pregnant mare from the 6th to the 15th week of pregnancy.

3) *Gonadotrophic and oestrogenic substances during normal pregnancy.*

a) *Gonadotrophin.*

Several years ago we made some determinations of the gonadotrophin content in urine and serum in three normal pregnancies. Our assay results have been published in papers by *Kühnel (1935)*, *Guldborg (1936)* and *Andersen (1938)*. The test animals were infantile mice in which the formation of corpora lutea was used as the basis for the estimation, and the values were given in mouse units (M. U.). (According to *Hamburger & Pedersen-Bjergaard (1937)* this mouse unit corresponds to 15 of the rat units employed in the *chorionic* gonadotrophin assays in the present work). Since the investigations (summarized in Fig. 4) did not consist of more than 57 single measurements the values recorded may not be taken as an absolute expression for the average normal gonadotrophin excretion in pregnancy. It is, however, remarkable that the curves in Fig. 4 showing two maxima agree closely with the excretion curves in the new cases of pregnancy (Fig. 5) in

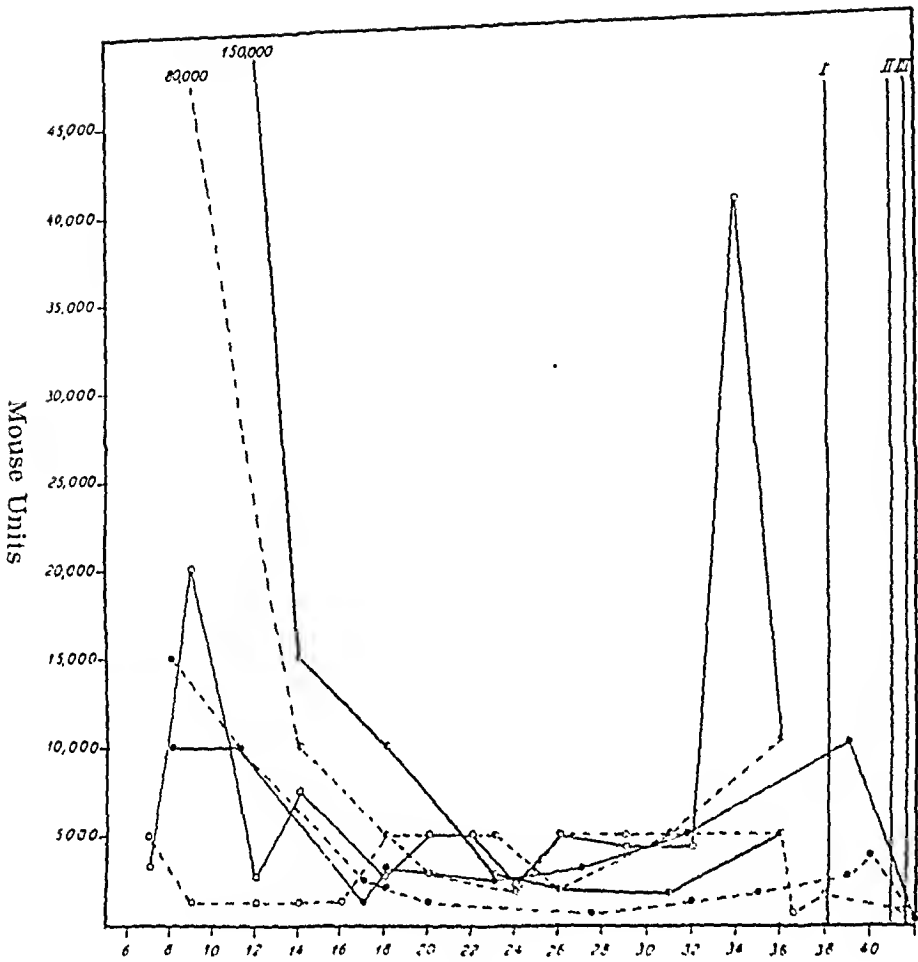


Fig. 4.

Duration of pregnancy in weeks.
Gonadotrophin content of the serum and urine in 3 normal pregnancies.

————: Urine, M. U. / 1000 ml.

-----: Serum, M. U. / 1000 ml.

- Mrs. PP, delivery on the 267th day.
- Mrs. QQ, delivery on the 286th day.
- Mrs. RR, delivery on the 291st day.

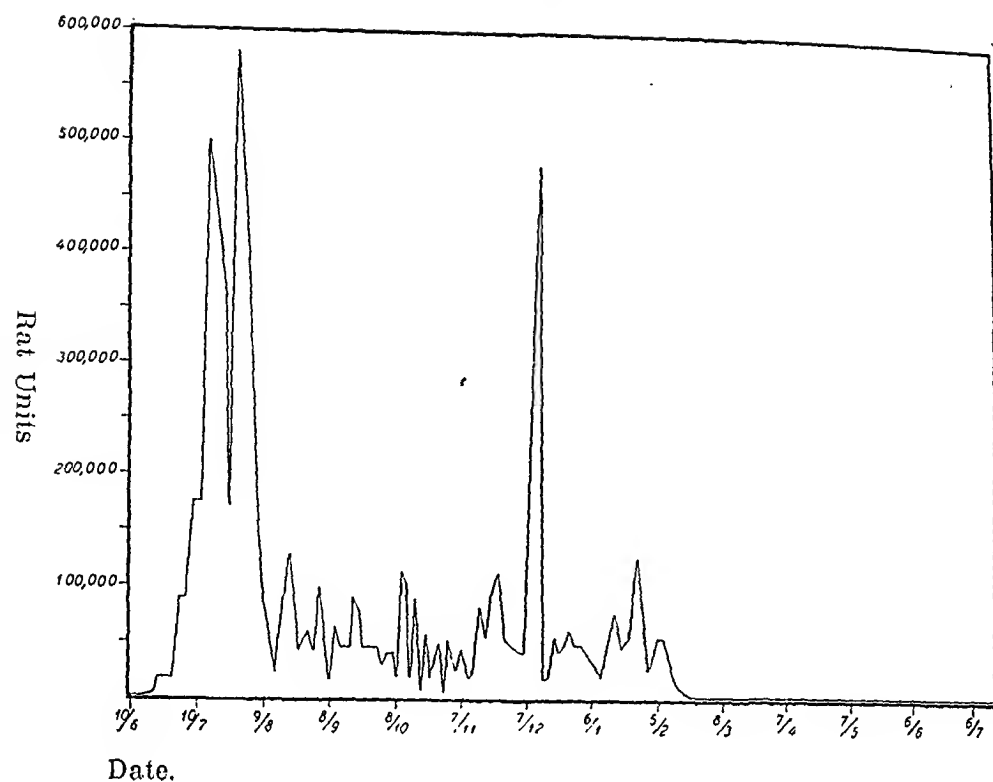


Fig. 5 a.

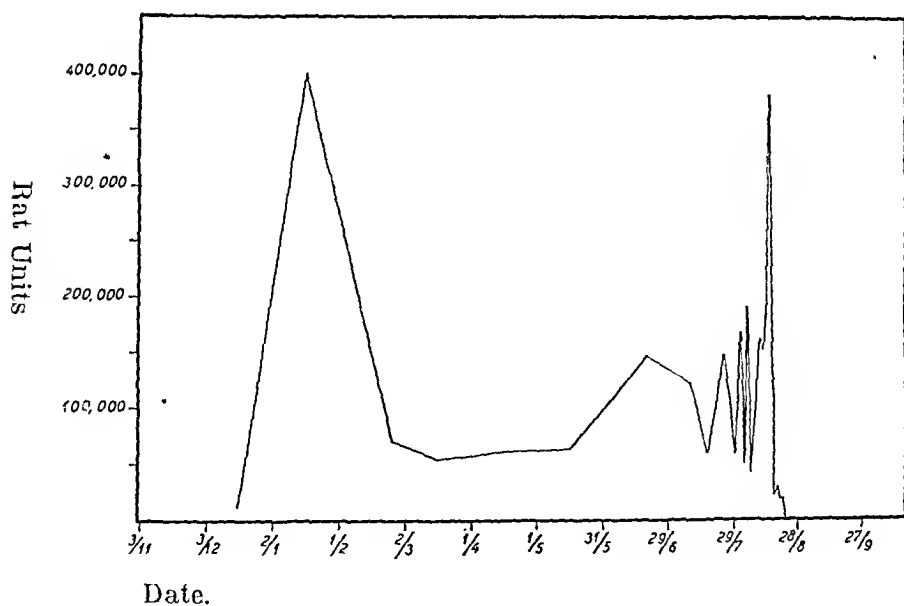


Fig. 5 b.

The gonadotrophin output (R. U./24 hrs.) in the same individual (Mrs. YY), measured during the two pregnancies:

- a. First pregnancy, 13/6 36 — 13/2 37.
- b. Second pregnancy, 6/12 41 — 18/8 42.

which two maxima occurred on 51'—67' day and on 183' day in the first pregnancy and on 42' and 253' day in the second pregnancy. After the first maximum with values up to 400.000 R. U.—600.000 R. U. per day, there was a rather marked fall in the gonadotrophin output, which in the following months varied between 20.000 and 100.000 R. U. per day. During the second maximum toward the end of the gestation the gonadotrophin output in both pregnancies measured about 500.000 R. U. per day. A late maximum is also found in the case of another pregnant woman (Mrs. NN, 31 years old, III-para) about 1 month before delivery (see Fig. 6). On the day of delivery the gonadotrophin output (Fig. 5) amounted to 20.000—30.000 R. U., and 10 days later it had fallen to the low level characteristic of the normal menstrual cycle.

The first maximal excretion of gonadotrophin, ascertained about 54 days after the beginning of the last menstruation, is in keeping with previous reports in the literature from *Brown & Venning* (1936) and *Evans, Kohls & Wonder* (1937), who reported the greatly increased output of gonadotrophin one month after the first missing menstruation, as demonstrated in 6 cases by titration of the 24-hour urine. *Hamburger* (1943) reported up to 900000 I. U. per liter of urine in normal pregnancies (2'—3' month). *Boycott & Rowlands* (1938) found the chorionic gonadotrophin content of the serum to reach its maximum in the same period. The second gonadotrophin maximum, appearing shortly before parturition, has not been described before in the literature. In this connection, it will be appropriate to quote the following remark from the paper by *Evans et al.*:

»Three erroneous schematic figures constitute the only charts known to us. These are the much copied figure of *Zondek* (1935) p. 358, fig. 140, the figure of *Frank* (1935) chart 5, and the figure of *Mazer & Goldstein* (1932). Graphs for individual cases have not appeared in the literature. It is undoubtedly due to their transient presence and to the lack of sufficiently careful titrations that the regular occurrence of these high urinary hormone values has been previously overlooked.«

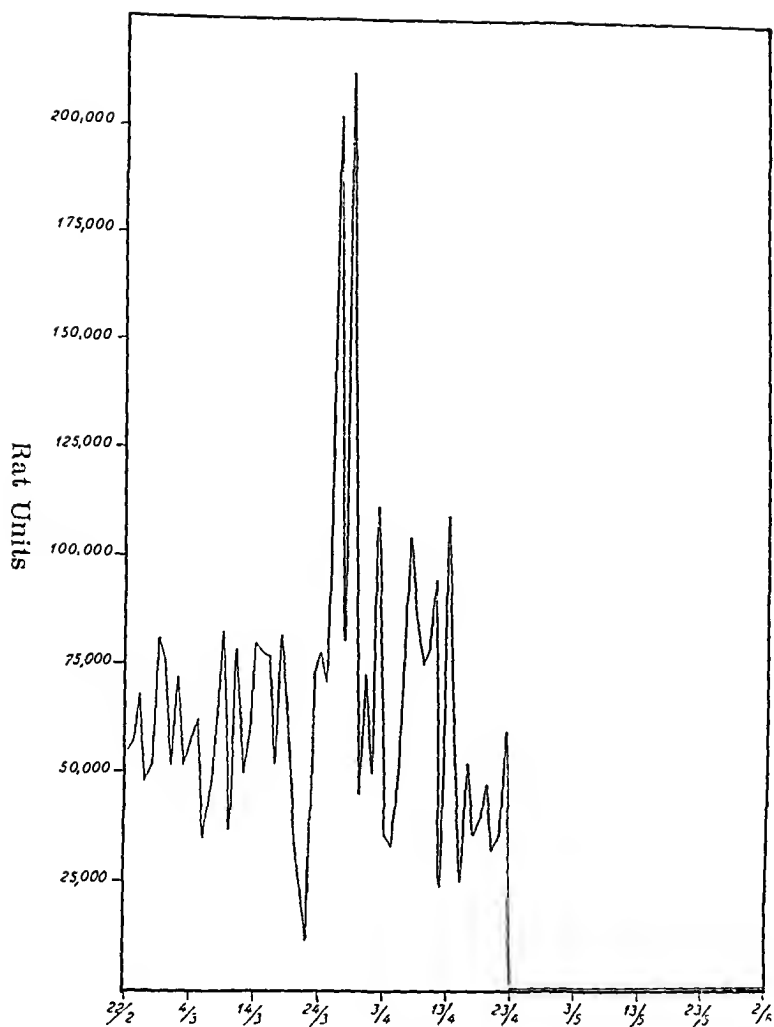


Fig. 6.

The gonadotrophin output (R. U./24 hrs.) in the last part of pregnancy (Mrs. NN III-para).

When previously nothing has been reported about the last gonadotrophin maximum, we think that this may be due to the fact that the last rise is of even briefer duration than the first one. In order to demonstrate the last rise it is essential to analyse all the urine throughout pregnancy. Omission of the analysis of merely one 24-hour urine may bring about that this last increase is not registered.

Variations in the chorionic gonadotrophin output within 24 hours.

It is generally supposed that the concentration of gonadotrophin in the first collected urine in the morning is higher than in the other outputs, therefore early morning urine is always required for the *Friedman* and *Aschheim-Zondek* tests. As no systematic investigations showing this fact seem to exist

Table 2.

*Gonadotrophin output in a case of pregnancy (Mrs. OO).
The 24-hour period is collected in 3 separate eight-hour portions.
Each figure represents the average value of 23 urine specimens.*

Collected from — to	R. U. total	R. U. in 1000 ml.	Volume of urine (in ml.)
10 p. m. — 6 a. m.	22100	39820	555
6 a. m. — 2 p. m.	27200	64762	420
2 p. m. — 10 p. m.	20200	54447	371
In 24 hours	69500	51634	1346

we have taken up the question for closer treatment. The daily amount of urine from Mrs. OO was collected during 23 consecutive days in 3 eight-hour portions, namely: 1) 10 p. m. — 6 a. m., 2) 6 a. m. — 2 p. m. and 3) 2 p. m. — 10 p. m. These 69 portions were examined separately. Thus it was found that within the 24-hour period a considerable variation in the 3 eight-hour portions often took place. As, however, it was not possible to decide with certainty whether this difference was due to technical variations or whether it was real, we have calculated the *average values* for each of the 23 eight-hour portions, these are recorded in Table 2. It will be noticed that no significant difference exists between the absolute amount of gonadotrophin excreted with the first portion (10 p. m. — 6 a. m. = »morning urine«) and with the third eight-hour portion, while on an average the portion collected from 6 a. m. — 2 p. m. contained the greatest amount. The difference, how-

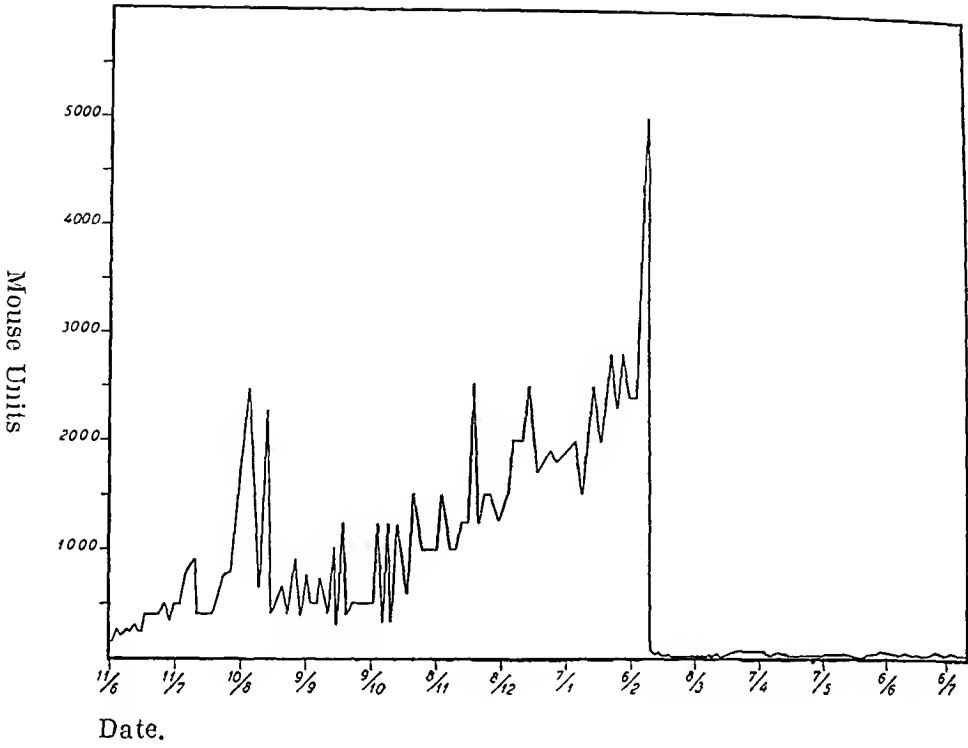


Fig. 7 a.

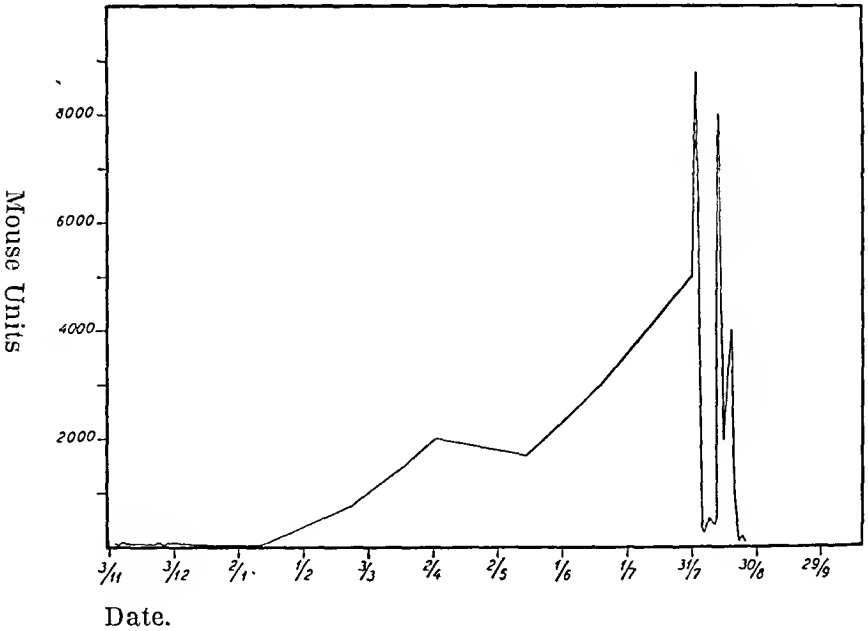


Fig. 7 b.

The oestrin output (M. U./24 hrs.) in the same individual (Mrs. YY) assayed in the two pregnancies:

- a. First pregnancy, 13/6 36 — 13/2 37.
- b. Second pregnancy, 6/12 41 — 18/8 42.

ever, is much greater if the *concentration* of gonadotrophin is calculated (R. U. per 1000 ml.). It is then found that the concentration of gonadotrophin in the «morning urine» is considerably lower than in the two other portions, an observation which is directly contrary to the view hitherto asserted. Although it cannot be excluded that the individual concerned in this respect represents an exception from the rule, the observation goes against the dogma of the advantages of the »morning urine« and incites to further investigations.

b) *Oestrogens.*

We have recorded the oestrin output of the same individual (Mrs. YY) during two periods of pregnancy, in Fig. 7 a and Fig. 7 b.

Fig. 7 shows a gradual rise in the oestrin output from about 200—300 M. U. per day at the beginning of pregnancy to 2000—3000 M. U. in the latter part of pregnancy, and about 5000—8000 M. U. in the days immediately before parturition. In this connection it may be appropriate to refer to the findings reported by *Moller-Christensen & Pedersen-Bjergaard* (1936), who in 58 women with normal delivery found the oestrin output on the day of parturition to vary from 1000—100.000 M. U. per liter of urine, with an average output of 20.000 M. U. Quite corresponding values were found for 17 parturients with primary uterine inertia, which does not indicate that the oestrin production is lowered in the case of this affection. The oestrin output here recorded in the 2 periods of pregnancy covers merely the free oestrogenic activity of the urine as measured directly without acid hydrolysis (Figs. 7 a and 7 b). In the case of the first pregnancy, 20 daily urines, scattered over the period of pregnancy, were examined both photometrically and biologically according to the principles given by *Cohen, Marrian & Watson* (1935) for their content of free oestrone, free oestriol, combined oestrone, and combined oestriol. The outcome of the latter studies has been reported previously by *Jensen & Pedersen-Bjergaard* (1942).

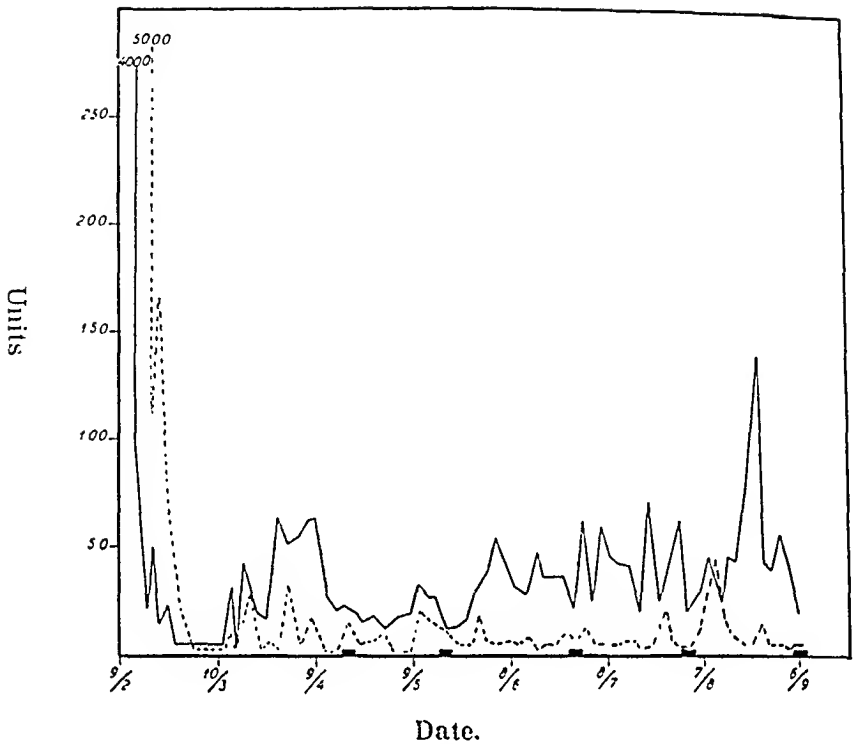


Fig. 8.

The output of oestrin and gonadotrophin during the first 7 months after delivery on 13/2 37.

—————: Oestrin M. U./24 hrs.

-----: Gonadotrophin R. U./24 hrs.

The dark fields signify the days of menstruation.

4) *Gonadotrophic and oestrogenic substances during lactation amenorrhea.*

The first period of pregnancy, which was concluded by delivery on 13/2—37 was followed by lactation amenorrhea for 65 days, after which a normal menstrual cycle appeared with cyclic periods of 35 days. The urine was analyzed through altogether 7 months after the parturition.

From Fig. 8 it will be noticed that the cyclic period, which prior to pregnancy amounted to 26 days, now lasts 35 days.

Further, the oestrin output is at a lower level after pregnancy than before, and its variations are not so characteristic as before pregnancy. Only in a couple of weeks, from 25/2—11/3, was the oestrin output as low as 5 M. U. per day, corresponding to a resting stage of the ovarian function. Then the oestrin output increased to 60 M. U. per day, and about 35 days after this increase, the menstruation reappeared.

During the 7 months after parturition the gonadotrophin output was rather low, generally below 10 R. U. per day.

SUMMARY

Urine from a period of forty eight hours from a young, healthy woman was collected continually throughout a period of 2 years, and 5 years later throughout a period of 10 months. These periods include 2 normal pregnancies.

The output of gonadotrophin and oestrin was determined and calculated in units per 24 hours. During the pregnancies the hormones were determined in the untreated urine, otherwise after tannic acid precipitation (gonadotrophin) and combined acid hydrolysis and carbon tetrachloride extraction (oestrin). Gonadotrophin was assayed on infantile female rats, oestrin on adult spayed mice.

Normal menstrual cycle.

The oestrin output was analysed in 24 cyclic periods, the length of which was 26 ± 3 days with 2 exceptions. 12 cyclic periods showed an oestrin output with 2 maxima, 8 periods showed only 1 maximum. The average for 22 periods showed the first maximum on the 12th day of the cycle, and another on the 22nd day. The oestrin excretion varied between 8 and 360 M. U. per 24 hours, the lowest output was found during menstruation.

The gonadotrophin was examined in 9 cyclic periods. The average excretion attained its maximum on the 11th day of the cycle and varied between 2 and 25 R. U. per 24 hours. No

regular correlation between the gonadotrophin and oestrin outputs was seen in the separate periods.

Pregnancy.

An increase in the gonadotrophin output was ascertained in both pregnancies before the first missing menstruation, namely on the 20th—21st day of the cycle. In the second month there was an enormous increase in the output of this substance, an equally high but briefer rise in the output appears towards the end of the gestation in all the three pregnancies examined. The oestrin excretion rises gradually to 3000 M. U. in the latter part of pregnancy and to even higher values in the days immediately before delivery.

Lactation Amenorrhea.

Twelve days after parturition the excretion of oestrin as well as gonadotrophin had fallen to hardly demonstrable values. The excretion remained at this level for two weeks.

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ON THE CONTENT OF CHORIONIC
GONADOTROPHIN IN THE FIRST MORNING
URINE AND IN AN AFTERNOON SAMPLE
COLLECTED FROM 19 PREGNANT
WOMEN

BY

CHRISTIAN HAMBURGER

The necessity of using early morning urine for the Aschheim-Zondek test has been emphasized in most publications on this reaction (e. g. *Zondek*, 1931, and *Aschheim*, 1933) because the concentration of the active substance was supposed to be highest in this urine. In accordance with these statements we have always asked for the first sample of morning urine for the Aschheim-Zondek and Friedman tests, and a negative reaction was considered to be of less significance if the test was performed with any other sample of urine.

Prompted by the observation of *Pedersen-Bjergaard & Pedersen-Bjergaard* (1948) that in a pregnant woman the urine collected from 10 p. m. to 6 a. m. during 23 days had on an average a lower concentration of chorionic gonadotrophin than each of the other two 8-hour samples, we have made the following examination.

From 19 healthy women in the 2nd to the 3rd month of pregnancy two samples of urine were collected within the same day, viz. the first morning urine and a sample from the after-

Table 1.

Case no.	Spec. gravity of	
	first morning urine	afternoon sample
1	1.020	1.010
2	1.005	1.016
3	1.010	1.018
4	1.020	1.016
5	1.020	1.010
6	1.010	1.020
7	1.020	1.022
8	1.018	1.014
9	1.020	1.020
10	1.004	1.014
11	1.010	1.004
12	1.018	1.024
13	1.020	1.010
14	1.030	1.018
15	1.020	1.010
16	1.020	1.024
17	1.018	1.026
18	1.016	1.010
19	1.004	1.002
<i>Average:</i>	<i>1.0159</i>	<i>1.0153</i>

The specific gravity of the first morning urine and an afternoon sample from the same day collected from 19 pregnant women.

noon. The specific gravity of the urines is listed in Table 1; in 10 instances it was higher in the morning urine than in the corresponding afternoon sample, in 8 instances the reverse was the case. The average sp. gr. was 1.0159 and 1.0153, respectively. Equal amounts of the homologous urines were pooled and the content of chorionic gonadotrophin was assayed in 78 immature rats (uterine and ovarian weights). The pooled morning urines contained 69.000 I. U. and the pooled afternoon urines 68.000 I. U. of chorionic gonadotrophin per litre.

Since these figures as well as the average values for the specific gravity must be regarded as equal it seems to be a matter of pure chance whether the first morning urine or a

later sample possesses the higher gonadotrophin concentration. Consequently there can be no reason to maintain the claim of the first morning urine for the biological pregnancy tests.

SUMMARY

The average content of chorionic gonadotrophin as well as the average specific gravity were found to be equal in the first morning urine and in an afternoon sample from the same day collected from 19 healthy pregnant women.

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ADRENALECTOMY AND ALLOXAN DIABETES

BY

J. FOKKENS AND S. E. DE JONGH

It is well known that in the field of carbohydrate metabolism certain relations exist between hypophysis and pancreas. Shortly after learning the possibility of provoking diabetes by means of alloxan we undertook in this laboratory a study of the changes which this artificially induced diabetes undergoes when the test rats are deprived of their hypophysis (*Gaarenstroom & de Jongh*, 1946, *Gaarenstroom*, 1947, *Gaarenstroom, de Jongh & Polder*, 1946, and *Gaarenstroom*, in press).

The symptoms of the disease (the high sugar-concentration in the blood and the excretion of sugar) were found to undergo a marked decrease, and might even entirely vanish, but, as the reaction of the animals upon the administration of sugar clearly showed, the metabolic disturbance of the diabetic animal remained the same. It also became clear that the improvement was, partly at least, a real one, and certainly not entirely a delusive impression caused by the circumstance that the animals had little appetite and consumed therefore less food than normal ones.

As it is generally assumed that there exists a similar relation between the adrenal cortex and the pancreas as between the hypophysis and the latter, we have now also investigated the effect exercised by the removal of the adrenals on the diabetic

condition provoked by the administration of alloxan. These experiments too were performed on rats.

Some data were already to be found in the literature on the influence exercised by adrenalectomy on alloxan diabetes. *Janes & Friedgood* (1945) reported that the presence of the adrenals is in rats indispensable for the development of a real alloxan diabetes, whereas *Goldner & Gomori* (1944) found that adrenalectomy suppressed in their test animal, the rabbit, the passing hyperglycaemia which otherwise would have manifested itself soon after the alloxan administration, but that it had no effect on the permanent diabetes which makes its appearance later on. The suppression of the preliminary hyperglycaemia was confirmed by *Kirschbaum et. al.* (1945) in experiments with rats, but these authors make no mention of what happens subsequently. *Janes, Dawson & Myers* (1946) found that adrenalectomy counteracts in rats the development of alloxan diabetes, but on account of experiments with paired feeding they ascribe this effect to the circumstance that the adrenalectomized rats consumed less food. Their conclusions are accepted by *Young* (1947), but it seems to us that they do not follow with sufficient certainty from their experimental data, and for this reason, and also because of the other contradictions in the literature, it seemed to us worth while to resume the study of this problem.

OWN INVESTIGATIONS

Our investigations consist of two series of experiments. In the first series the adrenals were removed before alloxan was given; in the second one the latter was administered before the removal of the adrenals. The alloxan was given in the form of a subcutaneous injection, the dose being 0.25 ml. of a solution of 75 mg. in 1 ml. 0.001 n HCl. The adrenalectomized rats (adults) were kept alive by means of desoxycorticosterone acetate (Doca) of which daily two doses each of 0.1 mg. were given. We controlled the body weight, and determined the amounts of urine excreted in consecutive periods of three

days; in the latter the sugar concentration and the amount of nitrogen were estimated. All animals received daily 11 gm. of the food we usually give them, and the less hungry animals too consumed this amount entirely.

Series I.

Alloxan was administered at the end of the first period of observation which, like the other ones, lasted three days. One

Table 1.

Estimations of quantity, sugar- and nitrogencontent of urine in rats; alloxan was given after the adrenalectomy.

	Body weight	Excretion in successive periods of 3 days each		
		urine in ml.	sugar in gm.	nitrogen in gm.
4 normal rats	1st day 189	1st per. 17	0	0.39
	5th day 188	2nd per. 17	0	0.41
	8th day 186	3rd per. 23	0	0.45
	11th day 188	4th per. 22	0	0.48
	14th day 186	5th per. 31	0	0.52
5 adrenal-ectomized rats	1st day 184	1st per. 19	0	0.40
	5th day 184	2nd per. 19	0	0.43
	8th day 185	3rd per. 22	0	0.44
	11th day 186	4th per. 25	0	0.47
	14th day 191	5th per. 21	0	0.47
5 normal alloxan rats	1st day 197	1st per. 23	0	0.49
	5th day 191	2nd per. 123	6.7	0.68
	8th day 179	3rd per. 124	5.9	0.88
	11th day 167	4th per. 124	8.0	0.98
	14th day 159	5th per. 94	5.6	0.90
7 adrenal-ectomized alloxan rats	1st day 198	1st per. 23	0	0.47
	5th day 193	2nd per. 35	0.7	0.49
	8th day 190	3rd per. 45	1.1	0.58
	11th day 189	4th per. 45	1.1	0.65
	14th day 185	5th per. 41	0.9	0.68

day after the alloxan administration the collecting of the urine was resumed. The rats were divided in four groups, viz. normal and adrenalectomized ones that received no further treatment, and served as controls, and normal and adrenalectomized ones that received an alloxan injection.

As the list of body weights given in table 1 shows, the amount of food was sufficient for the two groups of animals that received no alloxan. Of the alloxan rats the »normal« ones decreased more in weight than those whose adrenals had been removed. The amount of urine excreted by the adrenalectomized alloxan rats showed a slight increase, but that excreted by the »normal« alloxan animals a very strong one. The »normal« alloxan rats excreted on the average several grams of sugar per day, the adrenalectomized ones but a fraction of this amount: in fact, three out of the seven rats of this group excreted no sugar at all. The N-excretion too was in the »normal« alloxan rats much larger than in the adrenalectomized ones, in which it had, however, in comparison with that of the two groups of controls, also markedly increased.

Series II.

This set of experiments differed from the preceding one in two points. In the first place the alloxan was administered, as has already been mentioned, before the first observation period, whereas the adrenals were extirpated at the end of the latter; and in the second place the animals were finally loaded with sugar. To this end a 20 per cent dextrose solution was made of which 2 ml. were administered orally and 2 ml. an hour later intraperitoneally. The blood-sugar value was determined before the administration of the sugar and once more an hour after the intraperitoneal injection. The results are given in table 2.

It will be seen that table 2 confirms the results of table 1 with one restriction: the nitrogen excretion of the adrenalectomized alloxan animals is here hardly higher than that of the normal and adrenalectomized controls. The initial blood-

sugar values were on the average lowest in the adrenalectomized alloxan animals, but the values found in the untreated adrenalectomized animals can hardly be regarded as higher, for the difference appears to be within the limits of the probable error. In the »normal« alloxan rats the blood-sugar value reached, as was to be expected, a far higher level than in the normal controls. The loading with sugar provokes in the alloxan animals *always* a much higher increase than in the other ones, no matter whether the adrenals are present or absent and how widely, on this account, the initial values differ.

DISCUSSION

The experiments described above lead to the conclusion that the rule according to which the symptoms of diabetes provoked by alloxan are in the adrenalectomized animals less severe than in normal ones retains its validity when the adrenalectomized animals consume the same amount of food as the other ones. The improvement, moreover, is but spurious, for the ability to produce insulin is not restored. This appears at once when the animals are loaded with sugar, for in that case the originally widely differing blood-sugar values of the adrenalectomized alloxan animals and the »normal« alloxan animals are found to undergo a similar increase.

There is in all these points such a far-going parallelism between the effect produced by the removal of the hypophysis and that produced by the removal of the adrenals, that it may be asked whether the immediate cause of the changes brought about by these operations must not be sought in the adrenals, whose activity is, as we know, strongly influenced by the hypophysis.

If this problem is to be solved, we will first of all have to find out what part the removal of the adrenals plays in the alleviation of the diabetic symptoms. With regard to this question we fully agree with *Young*, who l. c. points out that the adrenal cortex may have a twofold influence; it may keep, as *Russell* (1943) suggested, the sugar oxydation within bounds,

but it also may favour the glyconeogenesis. Both kinds of activity would tend to create a condition as that reflected in our data in the nitrogen excretion.

The problem of the relation between the hypophysis and the diabetic condition must on account of these reflections be regarded as even more intricate than was originally supposed. The diabetogenic effect of the pituitary extracts, i. e. the induction of a lasting diabetic condition independent of the continuation of the injections, has generally been ascribed to the presence of growth-hormone in these extracts, but we have now come to the conclusion that the disappearance of the diabetic symptoms is not due to the elimination of this growth-hormone but to the absence of corticotrophic hormone! In the literature there are already some indications which point in the same direction.

Long et. al. (1940) worked with rats that had been made diabetic by extirpation of the pancreas, and found that administration of *cortical extracts* caused in these animals an increase of the blood-sugar values and of the nitrogen excretion. In cats that had been deprived of their adrenals as well as of the pancreas, *Long* (1936—37), however, did not find a return of the diabetic symptoms when they were injected with *a pituitary extract that was known to cause diabetes* in normal animals. This need not surprise us when we realize that the diabetogenic activity of the pituitary extract is, according to recent investigations, due to the presence of growth-hormone. In our laboratory *Gaarenstroom et. al.* (in press) too were unable to provoke diabetic symptoms in hypophysectomized alloxan animals by means of a growth-hormone preparation. *Houssay & Biasotti* (1938), on the other hand, obtained positive results in dogs that had been deprived of their hypophysis and pancreas, by administering simultaneously diabetogenic pituitary extracts and adrenal cortical extracts. This is comprehensible when we regard it in the light of the synergism which, according to *Russell* (1939), exists between the pituitary extract and cortical hormone in their action on the carbohydrate metabolism. *Bennett & Li* (1947)

observed in rats in which diabetes had been induced by means of alloxan, an increased excretion of sugar and nitrogen after the injection of corticotrophic hormone. The experiment that would decide the question of the real nature of the alloxan diabetes, the administration of corticotrophic hormone to hypophysectomized alloxan animals, has, as far as we know, not yet been performed. For the moment the interpretation given above seems to us, in the light of the available data, the most plausible one.

SUMMARY

1. In paired feeding experiments the excretion of water, glucose and nitrogen with the urine in adrenalectomized alloxan treated rats is considerably lower than in intact alloxan rats.
2. The order of sequence of adrenalectomy and alloxan injection is immaterial as regards to the results mentioned.
3. Adrenalectomy does not alter the bloodsugar rise which occurs after loading with glucose.

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BIOLOGICAL METHODS FOR DETERMINATION OF POTENCY OF GROWTH HORMONE PREPARATIONS

BY

TAGE KEMP

Two papers published recently in this journal by *Dingemanse, Freud & Uyldert* (p. 71, 1948) and by *Gjeddebæk* (p. 258, 1948) discuss the various biological methods for determining the potency of growth hormone preparations.

Dingemanse et al. propose to designate the growth hormone as chondrotrophin and prefer to speak of chondrotrophin rather than somatotrophin (Selye). The reason for introducing the new expression chondrotrophin is, however, not quite obvious. No evidence at all proves the existence of an independent chondrotrophic hormone or preparation, such a substance has not been isolated yet.

The typical many-sided effect of growth hormone preparations has, as regards hereditary pituitary dwarfism in mice, been proved many years ago by *Kemp* in a paper, published 1934, also demonstrating for the first time the possibility of using dwarf mice for potency determinations of growth hormone preparations. In this publication *Kemp* (l. c. p. 1855) emphasises: »... dass bei Zwergmäusen die Zufuhr von Wachstumshormon bedeutendes, wohlproportioniertes Wachstum hervorruft, das sich ziemlich gleichmässig auf alle Organe, u.

a. auch auf das Knochengerüst, indem die Röhrenknochen deutlich in die Länge wachsen, erstreckt«.

The versatile effect of growth hormone preparations on dwarfism in mice has been confirmed later on by *Kemp & Marx* (1937) and *Fonss-Bech* (1947). These investigators demonstrated the effect of growth hormone preparations*) in dwarf mice on total weight, on length (body length, tail length and total length), in skeletal development and on weight and structure of endocrine glands and other interior organs, on vitality and behaviour, basal metabolism and resisting power to cold. Later *Lykkegaard Nielsen* analysed the effect of growth hormone preparations on the ossification process and skeletal growth and *Helweg-Larsen* studied thoroughly the effect of the hormone on the individual cell, cell nucleus and cell-division, (the investigations by *Lykkegaard Nielsen* and *Helweg-Larsen* have not yet been published). Other collaborators at the Copenhagen Institute for Human Genetics investigated the embryology (*Francis*, 1944, 1945), the normal and pathological anatomy and physiology of the dwarf mouse (*Kemp & Marx*, 1936, *Francis*, 1945, *Bartels*, 1941, *Mollenbach*, 1941, and *Grunnet*, 1942).

Thus the anterior pituitary dwarf mice constitute a thoroughly studied rare and unique animal material for investigations into growth hormone problems.

Therefore it is surprising to see *Dingemanse et al.* write (l. c. p. 72): »We disagree with those authors who advocate other than skeletal indicators of chondrotrophin activity in non hypophysectomized animals. Even (hypopituitary) hereditary dwarf mice are not acceptable substitutes, though in these weight increments may be parallel with skeletal growth.« The reason for *Dingemanse et al.* holding this point of view must be that they are not sufficiently familiar with the aforementioned investigations.

Contrary to *Dingemanse et al.*, *Gjeddebæk* (1948) highly

*) For all more recent experiments the growth hormone preparation, *Phyol*, produced by Alfred Benzon Ltd., Copenhagen, was used.

recommends the plateaued female rats method for determining the potency of growth hormone preparations.

It has to be considered, however, that a relevant biological determination of the potency of a hormone preparation, a real biological standardization, cannot be carried out, without a standard preparation. The susceptibility of experimental animals as regards most biological reactions varies during the different seasons and during the series of years in accordance with the variation in internal and environmental factors in the animal or the strain of animals in question. *Dingemanse et al.* (l. c. p. 78) have used acetone dried powder of freshly frozen bovine hypophyses as a standard or reference preparation. *Fonss-Bech* (personal communication) has, however, considered the use of a standard preparation of this type impracticable, because he observed that the log dose-response curves for the ordinary growth hormone preparation and an acetone-precipitated standard preparation were not parallel.

According to our experience a quite satisfactory standard preparation of the growth hormone has not yet been produced. This is the reason why growth hormone potency determinations with various methods continued during several years cannot be compared without reservation. *Gjeddebæk*, however, tries to compare the results of potency determinations on 200 dwarf mice extended over a period of several years, with potency determination according to the plateaued female rats method on 40 rats (*Gjeddebæk* l. c. p. 258), extended only over a few months. *Fonss-Bech* has quite righteously compared the potency determination on 40 rats with the potency determination on 40 dwarf mice selected at random but in *chronological succession* to be able to compare two experiments as far as possible of equal duration. This circumstance has perhaps not been pointed out sufficiently by *Fonss-Bech*, probably because he has not imagined such a misunderstanding possible.

In 1946 *Kemp* defined a dwarf mouse unit (D. M. U.) of growth hormone as the smallest amount of growth hormone which on daily subcutaneous injections (6 days a week) for 3 weeks into dwarf mice with pituitary dwarfism, aged 4—7

weeks and weighing 5—7 g, produces an average increase in weight of 100 per cent.

The accuracy of the method depends naturally on the number of animals used for each potency determination. According to our experience during many years this method is quite satisfactory for potency determination of growth hormone and recently we have collected a comprehensive material of potency determinations on dwarf mice carried out within a rather limited period of time confirming this opinion. Of course this method may advantageously be supplemented or in the future even replaced by other methods, but for the present time it works gratifying. Certainly it will be an essential progress when a standard preparation fit for use is obtainable.

Fonss-Bech proposes to apply the dwarf mouse method with 2 weeks injection instead of 3 weeks, for routine potency determination. He demonstrates, however, (*Fonss-Bech*, l. c. p. 78, fig. 8) very clearly the greater accuracy obtained when using test periods of 3 weeks or longer duration instead of 2 weeks.

In our potency determination experiments we have always used a period of at least 3 weeks, in some experiments of 25 days or even longer. Applied in this way the dwarf mice method for potency determination of growth hormone, gives quite satisfactory results.

SUMMARY

The multifarious effect of the anterior pituitary growth hormone and the way it has been studied on dwarf mice are mentioned. The possibility of using dwarf mice for potency determination of growth hormone preparations is discussed.

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EFFECT OF THE SULFONAMIDES AND METHYL THIOURACIL ON THE BLOOD IODINE AND BLOOD CHOLESTEROL VALUES IN THE RAT

BY

OLAVI KINNUNEN

Determination of the effect of the goitrogenic agents on the function of the thyroid. There are certain methods which enable the investigator to obtain information of the effect of the goitrogenic agents, both with clinical material and animal experiments. Of such methods the following may be mentioned: histologic examination of thyroid preparations, continued measuring of the weight of the thyroid and of the whole organism, measuring the circumference of the neck, observations of clinical hypo- and hyperthyroid symptoms and the basal metabolism test.

In addition, valuable information may be obtained also from *determinations of the blood cholesterol and iodine content*. It has long been known (e. g. *Epstein & Lande*, 1922) that there exists a relationship between hypothyroidism and hypercholesterolemia or, respectively, between hyperthyroidism and hypocholesterolemia. This relationship has been held so regular that following the blood cholesterol level has been suggested as a means of studying the functions of the thyroid. According to *Glidea, Man & Peters* (1940), the possibility of hypothyroidism could be completely excluded if the blood cholesterol content is below 275 mg. per cent. Similarly it has been

shown that the blood cholesterol level is raised by thyroidectomy and lowered by a feeding of thyroid. Some workers have already availed themselves of this fact by following the blood cholesterol content in their investigations of the effects of the sulfa and thiourea preparations (Stoessel, 1944, Dunlop, 1945, Thyssen, 1947, and others).

It has also been clearly shown that in a study of the functions of the thyroid, valuable conclusions can be drawn from changes in the blood iodine content. This question has been recently discussed by Soisalo (1948), Unonius (1946) and Rouhunkoski (1948), so a brief reference may suffice here. It should be mentioned only that the blood iodine and cholesterol levels are inversely proportioned so that in clear cases of hypothyroidism the blood iodine values are generally low and in cases of hyperthyroidism high (cf. Elmer, 1938). However, the available literature contains no references to the effects of sulfa and thiourea preparations on the blood iodine level.

OWN INVESTIGATIONS

Outlining of the problem. The object of my investigations was to study the effect of certain sulfa preparations and of methyl thiouracil on the blood total iodine and total cholesterol level in the rat.

Material. Altogether 63 adult rats were employed in the experiments. The animals were of the special white strain of the Institute of Medical Chemistry. The weight of the rats at the start of the experiments was 200 ± 20 gm. During the experiment the animals were fed *ad libitum* with the standard laboratory fare. Water consumption was not measured. The daily dosage of the sulfa preparation (sulfaguanidine, sulfathiazole, or sulfanilamide) was 50 mg., and of methyl thiouracil 5 mg., per each 100 gm. body weight. The preparations were thoroughly mixed with the food. Rats receiving the same preparation were placed in the same cage, three in each. The duration of the experiment was 1, 3 and 6 weeks.

Determination of iodine and cholesterol. Determination of the blood total iodine was carried out by the *Leipert* method, as modified in this Institute (see *Rouhunkoski*, 1948). For the determination of blood cholesterol, the method of *Bloor*, likewise modified in this Institute (*Vesa & Kalaja*, 1939) was used. After all the rats in the cage were killed by decapitation, 3 ml. blood from each animal was taken for the iodine determination and 2 ml. for the cholesterol determination. Blood samples from animals belonging to the same group were then combined, and the results obtained therefore already represent mean values. The results are shown in table 1.

Preparation	Time: 1 week		Time: 3 weeks		Time: 6 weeks	
	Iodine per cent	Cholesterol mg. per cent	Iodine per cent	Cholesterol mg. per cent	Iodine per cent	Cholesterol mg. per cent
Controls	6.0	180	6.8	210	7.3	200
	7.4	210	6.9	190	6.7	205
Sulfaguani- dine	9.7	235	5.7	270	5.0	305
Sulfathia- zole	8.6	220	6.0	260	5.4	290
Sulfanila- mide	8.8	200	6.9	230	5.9	260
Methyl thiouracil	9.8	240	5.2	285	3.1	350
	11.3	280	4.8	330	3.8	325

RESULTS

The experiments, whose object was to ascertain the effect of certain sulfa preparations (sulfaguanidine, sulfathiazole, sulfanilamide) and of methyl thiouracil on the blood iodine and cholesterol levels of healthy adult rats, showed that all preparations first cause an increase of the iodine content of the blood, which, however, subsequently decreases and reaches values below the controls when the experiment has lasted at least 3 weeks. Those preparations, which caused the most pronounced increase in one-week experiments were found to lower most the iodine content in prolonged use. The high iodine content noted in the short-time experiments corresponds well to the period of

mobilisation of the iodine that has been stored in the thyroid (*Franklin et al.*, 1943, 1944), while the values obtained in the more prolonged series correspond to the hypothyroid state, developed during the experiment. The uniform and gradual rise of the blood cholesterol is well in keeping with a gradually developing hypothyroidism. The noted proportions in the effects of the different sulfa preparations correspond, for instance, to the results of *MacKenzie et al.* (1941, 1943) concerning the histologic changes produced by various sulfa drugs. Methyl thiouracil could be expected to have relatively more marked effect.

SUMMARY

Experiments were made with adult white rats in order to study the effects of sulfaguanidine, sulfathiazole, sulfanilamide and methyl thiouracil on the iodine and cholesterol content of the blood. It was found that:

- 1) in 6-week experiments the blood cholesterol showed a continuous increase up till 260—350 mg. per cent.
- 2) the blood iodine increased at the early stages of the experiment, but later the values began to fall, being ultimately up till 50 per cent lower than the normal values.

The rise of the cholesterol level corresponds well to a gradually developing hyperthyroidism. The early increase of the iodine level probably corresponds to the mobilisation of the thyroid-bound iodine, and its decrease to a development of hypothyroidism.

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THE SIZE OF THE PITUITARY BODY AND ITS LOBES AFTER THYROIDECTOMY AND AFTER PARATHYROIDECTOMY IN ADULT MALE AND FEMALE RATS

BY

SVEN ELOV BROLIN

Alterations in the size of the endocrine glands still play an important part in the study of the relations between the function and its morphological background. During the last few decades the size of the pituitary body under various conditions has been the subject of at least a couple of hundred investigations. Only in a few of these, however, has the size of the individual lobes been determined with reference to the effect of different factors. Our present knowledge is accordingly confined to the effects of inanition, pregnancy, castration, treatment with ergosterine, and exposure to cold. Particulars concerning the influence of these factors have been given by *Jackson* (1917) for inanition, by *Stein* (1934) for pregnancy, by *Brolin & Löfgren* (1947) for castration, by *Agduhr* (1932) for ergosterine, and by *Brolin* (1945) for exposure to cold.

Knowledge of the size of the anterior lobe has been of essential importance in studies of the structural correlate to the hormonal reactions after castration and exposure to cold. So far as the intermediate and posterior parts of the hypophysis are concerned, it has been possible in certain cases to de-

monstrate that the size of the pars intermedia undergoes changes the causes and significance of which are still unclear.

The question as to whether the morphology of the pituitary body is affected by thyroidectomy has been dealt with in an extensive literature. As early a worker as *Trautmann* (1916) gave almost forty references to the literature on this aspect, and *Altschule & Cooper* (1937) collected the results of more than fifty investigators, some of whom, however, had already been mentioned by *Trautmann*.

How the total size of the hypophysis changes in the rat after *thyroidectomy* would seem to have been cleared up so far as the males are concerned. In them an increase occurs in the weight of the pituitary body, according to the concordant findings of *Hammett* (1923, 1926), *Zeckwer, Davison, Keller & Livingood* (1935), *Lebedewa* (1936), *Zeckwer* (1937, 1938), *Smelser* (1939), *Turner & Cupps* (1940) and others. This increase has been recorded for adult as well as infantile male rats.

The size of the pituitary body after thyroidectomy increases in infantile female rats as well according to *Hammett* (1923, 1926), *Zeckwer, Davison, Keller & Livingood* (1935), *Zeckwer* (1937, 1938), and *Bischoff* (1941). In the case of adult female rats, however, there is a divergence of views as to the size of the gland after thyroidectomy. Whereas *Hammett* (1923, 1926) contends that a decrease takes place, *Evans & Simpson* (1930) as well as *Turner & Cupps* (1940) claim that there is an increase.

The effect of *parathyroidectomy* does not seem to have been investigated to any great extent. *Hammett* (1923, 1926) states, however, that the size of the pituitary body is unaffected after this operation. What role is played by the parathyroid glands in these changes in size of the rat's hypophysis after thyroidectomy has not been elucidated. When performing this operation the authors cited have in the majority of cases extirpated the parathyroid glands and no intact parathyroid tissue has been found at the end of the experiment.

A survey of the available literature data shows that the

total size-changes of the pituitary body after thyroidectomy have not yet been clarified in all respects, and any significance the parathyroid glands may possess in that connection has remained unnoticed. Moreover, our knowledge of these alterations in size must be denoted as incomplete so long it has not been made clear to what extent they fall on the different parts of the organ. Hence, in spite of the time-consuming nature of the quantitative method, it has been considered highly desirable to make an analysis by modern statistical methods of the size assumed by the pituitary lobes after thyroidectomy and after parathyroidectomy in male as well as female rats.

MATERIAL AND METHODS

The animal material employed consists of 108 rats, which were 9 months old at the beginning of the experiments. In a few cases the age was 8 months, in some others it approached 10 months. Control animals of the same age and from the same litters were consistently used. The rats were fed on a fare consisting of crushed rye, maize, coarse bread, milk, liver and lungs.

As the correlation that exists between animals from the same litters may be suspected to constitute an appreciable source of error at the estimation of the experimental results, every effort was made to get the animal material distributed over as many litters as possible. In a great number of cases, therefore, animals of the same sex were taken from the same litter for both thyroidectomy and parathyroidectomy as well as for control purposes. The males are from 17 litters and the females from 19, in most cases common litters for both sexes. A survey of the ways in which the individual animals within the different experimental groups were used is given in Table 1. This table also gives the mean body weights of the animals before the beginning of the experiment and at its end. From the former data it will be seen that no difference in weight between control and test animals can be considered to exist.

Thyroidectomy was performed, without removing the parathyroid glands at the capsule of the thyroid, by a technique previously described (*Brolin, 1945*). The presence of normal parathyroid tissue could be verified with few exceptions at the end of the experiments. The parathyroid glands were removed with a fine thermocautery, after brushing a 1 per mille adrenaline solution on the surface of the thyroid. In this way they

Table 4.

	Male Rats			Female Rats		
	Normal	Thyroidect.	Parathyroidect.	Normal	Thyroidect.	Parathyroidect.
Number	28	18	10	24	15	13
Weight before beginning of exp.	200 ± 7.0	202.3 ± 6.0	201.5 ± 6.1	153.3 ± 4.5	154.9 ± 5.3	150.5 ± 6.0
Weight at end of exp.	197.6 ± 7.1	205.1 ± 9.4	176.8 ± 10.0	149.8 ± 5.2	155.5 ± 4.7	141.8 ± 7.3

Weights of the animals divided into control and experimental groups.

are known to be much more easily recognized. If both the glands occurring in the rat were not immediately found, one of them could in some cases be discovered behind the oesophagus and be extirpated. In a couple of cases in which both the glands could not be traced, thyroidectomy was done instead. After the operation the animals were allowed to live between 5 and 6 weeks and were then killed by severance of the cervical vessels under ether narcosis.

The hypophyses were worked out of their capsule under a lens and were weighed on torsion scales having controlled readings to 0.1 mg. After fixation in Susa they were placed in 90 per cent alcohol, then embedded with dioxane as intermediate in paraffin and cut on a sledge microtome into a series of 4 μ thick slices. Every fourth of these sections was taken and stained according to the technique of *Martins (1933)*.

Volumetric reconstructions of the pituitary lobes were made by projecting the preparations on paper to a magnification of 50.5 times, whereupon the individual lobes were sketched in, cut out and weighed. The weights of the individual lobes were then estimated from the weight of the hypophysis and the relation between the paper weights of the lobes. Use as in this investigation of a section at every 16th μ gives an insignificant error percentage, as explained in an earlier paper (Brolin, 1945). The method presupposes, among other things, the use of paper that is of very even quality in regard to thickness. From the paper used 200 specimens were taken for determination of its thickness, which did not deviate in any case by more than 0.5 per cent from the mean.

In the statistical calculations, besides the classical standard error calculation, use has been made of analysis of variance and calculations with the t distribution.

RESULTS

The calculations are based partly on the absolute weights of the whole glands and their lobes, partly on the proportional distribution of the hypophyseal weights among the lobes. Thus, the *size-values* for the individual lobes were calculated as (*a*) absolute figures and as (*b*) relative figures indicating the size of the lobes in per mille of the total pituitary weight. By means of an analysis of the per mille values it was thus possible to check the accuracy of the results, which can be considered also to apply to the absolute size-values.

To ascertain with what degree of probability the size-values obtained could have arisen at random in a homogeneous population essentially unaffected by the experiments, evaluations have been made with the aid of an analysis of variance (Table 2). The males with their control groups, both the experimental groups together and the females were then each brought together and the quotients of the inter- and intra-class variations were calculated.

For the males this analysis gave variance ratios that both as regards the whole gland and its anterior lobe could only in very ex-

Table 2.

	Pituit. Body			Ant. lobe 1) Weight 2) Per mille			Int. lobe 1) Weight 2) Per mille		Post. lobe 1) Weight 2) Per mille	
	Number	Variance Ratio	Proba- bility	Number	Variance Ratio	Proba- bility	Variance Ratio	Proba- bility	Variance Ratio	Proba- bility
♂ N	28	12.7	$p < 0.1$	28	1) 14.0 2) 7.09	$p < 0.1$ $p > 0.1$	1) 5.76 2) 1.82	$p < 1$ $p < 20$	1) 0.32 2) 8.32	$p > 20$ $p < 0.1$
♂ T	18			18						
♂ P	10			10						
♀ N	24	3.01	$p < 5$	24	1) 3.75 2) 3.53	$5 > p > 1$ $5 > p > 1$	1) 1.14 2) 2.40	$p > 20$ $20 > p > 5$	1) 2.21 2) 7.42	$20 > p > 5$ $1 > p > 0.1$
♀ T	15			14						
♀ P	13			12						

Analysis of variance. The different p-values denote the percentage probability of the variance ratios being exceeded by mere chance. Normal, thyroidectomized and parathyroidectomized rats are indicated by N, T and P.

Table 3.

	Mean ♂ N	Mean ♂ T	Diff. of means ♂ N — ♂ T	t- value	Proba- bility %	Mean ♂ P	Diff. of means ♂ N — ♂ P	t- value	Proba- bility %
Pituit. body mg	6.15 ± 0.25	7.86 ± 0.35	+ 1.71 ± 0.44	3.87	0.01	5.59 ± 0.23	- 0.56 ± 0.33	1.26	21
Ant. lobe mg	4.84 ± 0.22	6.42 ± 0.30	+ 1.58 ± 0.38	4.23	0.01	4.34 ± 0.21	- 0.50 ± 0.30	1.25	21
Int. lobe mg	784.1 ± 6.2	815.6 ± 8.3	+ 31.4 ± 10.4	3.10	0.2	774.3 ± 8.1	- 9.8 ± 10.2	0.85	39
Post. lobe mg	0.548 ± 0.025	0.670 ± 0.033	+ 0.122 ± 0.042	2.98	0.3	0.537 ± 0.028	- 0.011 ± 0.038	0.24	81
mg	89.4 ± 2.6	86.1 ± 3.2	- 3.3 ± 4.1	0.79	43	96.2 ± 3.8	+ 6.8 ± 4.6	1.39	16
mg	0.765 ± 0.033	0.762 ± 0.050	- 0.003 ± 0.060	0.06	95	0.715 ± 0.018	- 0.050 ± 0.038	0.87	38
mg	126.6 ± 5.2	98.4 ± 5.6	- 28.20 ± 7.6	3.59	0.04	129.6 ± 5.8	+ 3.0 ± 7.8	0.31	76
	Mean ♀ N	Mean ♀ T	Diff. of means ♀ N — ♀ T			Mean ♀ P	Diff. of means ♀ N — ♀ P		
Pituit. body mg	8.08 ± 0.42	8.72 ± 0.55	+ 0.64 ± 0.69	0.92	36	6.79 ± 0.60	- 1.30 ± 0.73	1.80	7.2
Ant. lobe mg	6.86 ± 0.39	7.26 ± 0.51	+ 0.40 ± 0.64	0.62	54	5.33 ± 0.54	- 1.53 ± 0.67	2.28	2.3
mg	843.0 ± 7.5	851.1 ± 8.4	+ 8.1 ± 11.3	0.69	49	815.4 ± 11.2	- 27.6 ± 13.5	2.08	3.8
Int. lobe mg	0.546 ± 0.028	0.528 ± 0.029	- 0.018 ± 0.043	0.42	67	0.482 ± 0.028	0.064 ± 0.40	1.46	14
mg	69.1 ± 3.0	64.7 ± 4.2	- 4.4 ± 5.2	0.87	38	78.7 ± 6.1	+ 8.6 ± 6.8	1.60	11
Post. lobe mg	0.699 ± 0.026	0.615 ± 0.035	- 0.084 ± 0.044	1.97	4.9	0.656 ± 0.25	- 0.043 ± 0.036	1.06	29
mg	90.8 ± 4.6	76.1 ± 3.3	- 14.7 ± 5.6	2.22	2.6	106.4 ± 6.1	+ 15.6 ± 7.6	2.00	4.6

The size-values of the pituitary body and lobes in different control and experimental groups. Normal, thyroidectomized and parathyroidectomized rats are indicated by N, T and P. The probability level has been calculated with *t*-analysis that the differences with their probable errors may appear at random in samples from a common population. The number of figures are in accordance with customary practice determined by the two figures of the probable error.

ceptional cases be obtained in samples from a homogeneous population. If a limit is drawn at one of these quotient values, this probability level would be reached or exceeded in one case out of a thousand. Under these circumstances it may be considered with a very high degree of probability that the weights of the hypophysis and of its anterior lobe had been affected in one of the experiments or possibly in both of them. Any further direct determination of the probability of different populations being concerned is scarcely compatible with the theoretical foundation of the analysis made. In so far as differences appear in the weights of the posterior lobe they may presumably be attributed to chance. On the other hand, the quotients of variance for the intermediate lobe represent a probability level that lies below 1 per cent. For the proportional size of the lobes the probability levels for the anterior and posterior lobes lie at about 0.1 per cent, and for the pars intermedia at about 20 per cent. Such displacements in the per mille figures for the anterior and posterior lobes would hardly occur within a homogeneous population. Perhaps it ought to be pointed out that if the per mille value for the anterior lobe alters considerably that for the posterior lobe will thereby be altered, even if the absolute weight of the latter lobe remains unchanged. The fact is that the per mille figure for one of the lobes can be regarded as an index to the aggregate size of the two others.

In the *females* the variance ratios of the hypophyses and the anterior lobes represent probability levels below 5 per cent. The ratio for the per mille value of the posterior lobe corresponds to a probability level between 0.1 per cent and 1 per cent. Provided the average absolute size of the posterior lobes is equal within the three groups, it may be taken that there is only a very small degree of probability in favour of the aggregate relative sizes of the anterior lobe and the pars intermedia having been derived from a common population.

The changes that after the analysis of variance may be expected to have occurred in certain of the size-values of the pituitary body cannot be exactly defined without a further statistical analysis. The reduction of the material has therefore been supplemented by the application of recognized statistical methods, including a *t* analysis. The last-mentioned method is especially suitable when comparisons between small populations are concerned, as it ensures a more trustworthy evaluation of the probability of their having by chance been selected out of the same population. The results of these calculations have been brought together in Table 3, which also gives the mean values and probable errors of the absolute and relative size-values, the differences and their probable errors as well as the corresponding *t*-values.

In the *males* it is found that after *thyroidectomy* the pituitary body undergoes a significant increase in weight. The increase depends essentially on a statistically significant enlargement of the anterior lobe. No doubt the pars intermedia also increases in size, and the *t*-value obtained here is only got by chance in 0.3 per cent. No change has admitted of being demonstrated in the absolute size of the posterior lobe. The relative size of the anterior lobe increases after thyroidectomy and shows a significantly larger per mille value, while the per mille value for the intermediate lobe appears to remain unaltered. In consequence of the relative increase of the anterior lobe the per mille figure for the posterior lobe shows a statistically significant diminution.

No evidence that parathyroidectomy affects the absolute or relative size-values of the pituitary body or its lobes has been obtained as a result of the probability evaluations undertaken. As intact parathyroid tissue was left behind in the majority of cases after thyroidectomy, and as no influence from parathyroidectomy was discernible, it has not been possible to find any other cause of the size-changes undergone by the hypophysis and its lobes than the extinguished function of the thyroid.

In the *females* no demonstrable effect of *thyroidectomy* has been registered on either the absolute or the relative size-values of the pituitary gland. The whole gland and its anterior lobe show an insignificant increase that is smaller than its probable error. *Parathyroidectomized* female rats have lower size-values both for the whole hypophysis and for the anterior lobe, and the diminution can be denoted as statistically probable. Probability levels lying at 7.2 per cent and at 2.3 per cent respectively have been here obtained. The relative size of the anterior lobe, like the latter's absolute size, shows a lower value in the parathyroidectomized females. The *t*-value in question corresponds to a probability level of 3.8 per cent.

In order to inquire further into the probability value of the weight-differences obtained, the size-differences have been calculated within each litter between the pituitary bodies of

the parathyroidectomized females and those of the control females. When there were several control-animals in the same litter, one was used for the mean value of these in common. The mean value of the diminution was estimated in this way at 1.39 ± 0.54 mg. and the corresponding value for the anterior lobe at 1.56 ± 0.56 mg. In the former case the diminution was 2.5 times and in the latter 2.8 times greater than its probable error. No alteration in the size of the pars intermedia could be discovered. Nor is there probably any change in the absolute size of the posterior lobe, for the difference, 0.043 mg., is slightly larger than its probable error. On the other hand, the per mille value for the posterior lobe shows a statistically probable increase that may be considered to mirror the above-mentioned diminution of the anterior lobe.

The experimental results exhibit a remarkable difference on a comparison between males and females in that thyroidectomy has not had any demonstrable effect on the pituitary size-values in the females. Consequently it has not been possible to verify earlier statements either of a decrease or of an increase in the total weight of the hypophysis in females. The absence of an increase in size does not lend any support to earlier experimental results pointing to a decrease in the size of the gland, since these results are based on experiments with combined thyroidectomy and parathyroidectomy and the decrease can then be conceived as due to the removal of the parathyroid glands.

As accessory parathyroid glands occur in certain types of rats, it may be questioned whether a pronounced parathyroid insufficiency has been the sequel of the operations performed. After interventions of the kind in question a diminution of the body weight has been registered that points to their not having been without effect. The reduction in body weight has been statistically examined by calculating for each animal the weight differences at the beginning and end of the experiments, whereupon the mean values and probable errors of the differences have been evaluated. The results disclose that in the males there is a decrease in weight of 24.7 ± 8.7 gm. and in

the females of 8.8 ± 3.1 gm. Hence the difference is 2.8 times greater than its probable error in both cases. The diminution in body weight is not due to the explorative cervical incision that is also done in thyroidectomy. After the latter operation, which is more time-exacting and extensive, there does not arise any weight reduction than can be registered at the end of the experiment (see Table 1).

The experiments undertaken show, in agreement with earlier reports in the literature, that the pituitary body of male rats increases in size after thyroidectomy. Examination of the size relations of the different lobes discloses that there is an augmentation in the weight of both the anterior lobe and the pars intermedia, while the posterior lobe remains unchanged in weight. The relative size of the anterior lobe increases. In females no evidence could be found of thyroidectomy having any effect on the size of the hypophysis or its lobes.

Parathyroidectomy does not seem to affect this gland in the males but in the females brings about a probable decrease in the absolute size of the gland and of its anterior lobe.

DISCUSSION

Some points of view on the connection between changes in the cell picture and in the size of the anterior hypophysis were submitted earlier by *Brolin* (1945), *Brolin & Theander* (1946) and *Brolin & Löfgren* (1947). The presence of a cell-transforming process may be expected, with origin of basophil cells from other kinds of cells and then probably the chromophobes. The basophiles hypertrophize and, when increased demands are made for cell-activity, they vacuolize and collapse. Micro-morphologically, the reactions of the basophil cells to thyroidectomy and exposure to cold are identical and may be considered on strong grounds to reflect the production of thyrotrophic hormone. In the cell-transforming process two elements may be distinguished which affect the size of the anterior lobe in two directions. Thus, this increases in size when large basophiles arise and becomes smaller when the supply of

these cells diminishes on account of their destruction. On the basis of these views the increased size of the anterior lobe in thyroidectomized males can be put into connection with the origin of the large basophilic thyroidectomy cells: Accordingly numerous, large, basophilic and vacuolized cells occur in the microscopic preparations. The failure of an increase in size to appear in females may depend upon a more active destruction of the basophiles in them.

In view of the agreement in the cytological reactions a comparison between thyroidectomy and exposure to cold is of interest. In the latter case there occurs a significant diminution in the size of the anterior lobe in both male and female rats (*Brolin, 1945*). Which of the sexes exhibits the greater diminution has not been determined, but in two series of experiments the numerical values of the decrease were greater in the females. In these experiments the anterior lobe of the pituitary body responded to the heavy demands imposed upon it by an increased incrtion of thyrotrophic hormone, at the same time as there was an increased consumption of the cell material. Whether the destruction of cells is greater after thyroidectomy or after cold exposure, is not clear. After thyroidectomy the hypertrophy and vacuolization of the basophiles seem to be more pronounced. Here, however, the microscopical preparation does not visualize a course but presents, so to say, a picture of a limited interval of time. It is unknown whether the pituitary bodies would become larger or smaller after thyroidectomy than after exposure to cold if a number of necrobiotic, vacuolized and large basophiles should disappear.

To account for the increased size of the pars intermedia after thyroidectomy of male rats the tempting explanation cannot be employed that the behaviour of this ought to be analogous to that of the anterior lobe, since both these organs belong to the glandular portion of the hypophysis. Analogous reactions of this kind cannot be postulated because after castration of male rats, the increase in size of the anterior lobe is not accompanied by a corresponding enlargement of the pars intermedia.

The possibility that the reactions of the anterior lobe may be dependent on the pars intermedia can be excluded, for if rats whose posterior and intermediate lobes have been ablated are subsequently thyroidectomized, this does not prevent typical thyroidectomy cells from arising in the anterior lobe (*Brolin*, 1947).

That changes in the metabolic state of the body may effect the size of the pars intermedia, is a possibility that deserves attention. To cold exposure, which brings about an altered metabolism, the pars intermedia responds by a statistically significant diminution in size (*Brolin*, 1945). *Jackson* (1917) likewise reports that the pars intermedia becomes smaller after inanition. Administration of ergosterine increases the size of the pars intermedia in mice (*Agduhr*, 1932). Lastly, it may be mentioned that the intermediate pituitary seems to diminish after castration of infantile female rats (*Brolin & Löfgren*, 1947). Among other metabolites coming in question in the experimental conditions mentioned it is perhaps justifiable to refer to derivatives of cholesterine, which presumably show an altered metabolism. For the present, however, it is not possible to advance other than speculative and hypothetical views on the size-conditions of the pars intermedia. Nor is it possible on the basis of our present knowledge to fit into any causal connection the statistically probable diminution of the hypophysis and its anterior lobe that occurs after parathyroidectomy of female rats.

With reference to the differences in the size of the pituitary body in the two sexes, it may be added that these do not merely consist of different mean values for the size of the whole gland and for its anterior lobe, for the females show a wider range of variation. With regard to the intermediate and posterior lobes no such sex-difference appears in the standard deviation. This fact has been observed by me in several earlier series of experiments, at which it also emerged that sex-differences in standard deviation are smoothed out after castration.

SUMMARY

Concordant reports in the earlier literature are to be found to the effect that the pituitary body as a whole of male rats increases in size after thyroidectomy, while there is a divergence of views as to its size in female rats.

In earlier investigations account has not been taken of the different lobes and insufficient attention has been paid to the role played by the parathyroid glands after thyroidectomy.

In the present study thyroidectomy alone and parathyroidectomy alone have been carried out on adult rats of both sexes. Besides the size of the pituitary body, the absolute and relative sizes of the lobes after volumetric reconstruction have been analysed by statistical methods.

Thyroidectomy is followed by an increase in the total size of the hypophysis in the males, the anterior and intermediate lobes likewise showing a significant enlargement. The relative size of the anterior lobe increases and that of the posterior decreases, although the latter's absolute size remains unaltered. In the females it could not be demonstrated that thyroidectomy had any effect on the size of the pituitary body or its lobes.

After parathyroidectomy the pituitary glands of the males seem to be unaffected while those of the females show a statistically probable diminution of the whole organ and of its anterior lobe. In concordance with this, the size of the posterior lobe presents a relative diminution in size but no absolute change.

Males and females exhibit differences not only in the form of different mean values for the whole hypophysis and its anterior lobe but also in the fact that the dispersion of the individual observations is wider in the female rats.

Some points of view are submitted concerning the role played by cytological factors in the pituitary size-changes.

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EXPERIMENTALLY INDUCED INTERSEXUALITY IN MICE

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By experimentally induced intersexuality is understood in this paper structural abnormalities in the urogenital system arising under the influence of various substances upon the embryo. The abnormalities that have sometimes been observed in the offspring of crosses between various races of animals, e.g. in some insects, as described by *Goldschmidt*, are therefore beyond the scope of this publication.

A considerable number of animal species have been used for experiments in the embryo. Suitable objects are the eggs of birds, because in those the embryos are at every desired stage of their development within easy reach, and because the treatment may be applied even before the embryo is formed.

This report is on experiments performed on mice and accordingly the considerations attached to the results will refer to mammals.

The work of *A. Raynaud* (1942) deals especially with experimentally induced intersexuality in mice. Its particular importance is due to a detailed account of the normal course of development of the urogenital system in the test animal. Without a sufficient knowledge of the histiogenesis no correct evaluation of the abnormalities in the test animals is possible.

Here follows a summary of the main points of the course of development.

- 1^o. The first rudiment of the sexual gland: the gonad, becomes visible on the 9th day of development; the differentiation into ovary occurs on the 11th day and into testis on the 12th day.
- 2^o. The so-called heterologous sexual ducts, in the male the Müllerian ducts, in the female the Wolffian ducts, begin to involute on the 15th or 16th day.
- 3^o. Up to the 17th day of development the urogenital sinus behaves in both sexes alike. After that day a difference between male and female begins to manifest itself. In the male the sinus yields the urethra; in the female it gets divided by a frontal wall into a ventral part (the urethra) and a dorsal part (the distal part of the vagina).
The proximal part of the vagina, which connects the distal part with the uterus, is formed from the so-called sinus cord, which extends from the dorsal side of the sinus roof to the place of union of the Müllerian ducts.
- 4^o. Whereas the Müllerian ducts in the male disappear entirely, the male homologues in the female may leave some residues, e.g. in the form of prostate rudiments on the ventral and lateral side of the urethra near the neck of the bladder.

The descriptions that have been given of the development of the urogenital system and especially of the development of the sinus urogenitalis in different animal species, do not fully agree with each other and several points are not yet clear. It is doubtful therefore whether Raynard's description is applicable to other animals (rat; guinea-pig). It is even possible that not all races of mice behave alike.

For the induction of intersexuality hormone-preparations were used, i. e. substances with oestrogenic and androgenic properties.

Whereas for bird-embryos oestrogens are less toxic than androgens the inverse is true for the embryos of mammals.

Oestrogens prove to be toxic to them, and the results that can be obtained with these substances are therefore of little importance in comparison with those that are obtained with androgens. The following report is confined to a study of androgens.

The methods that have been applied by the various investigators differ considerably. The most commonly used one is that in which females are injected subcutaneously at various stages of their pregnancy with varying doses of androgens, usually with testosterone propionate. Occasionally injections were given into the amnion cavity (Dantchakoff in guinea-pigs). Marsupian embryos can be directly treated at a stage when they are still little differentiated.

The total amount of androgen that has been used in the experiments with mice and rats varies considerably and is of the order of 5—50 mg. Sometimes even much higher doses have been applied. Injections during the first 9 to 15 days of pregnancy are often fatal to the embryos of rats and mice. More advanced embryos are more resistant.

Other authors used to investigate intersexuality immediately after birth or else within a few days; only in a few cases are autopsies of animals described after they had reached maturity.

Details due to differences in method, dosage, kind of test-animals and time of dissection are irrelevant here. The following survey of the general results obtained by the earlier authors will be sufficient.

1. No change in the structure of the ovary was ever observed.
2. The Müllerian ducts always develop normally. Very large quantities of androgen may slightly disturb the structure of the distal part of the duct.
3. Parts of the Wolffian ducts may, according to circumstances persist, yielding epididymes, ejaculatory ducts and seminal vesicles.
4. The most characteristic anomalies have been observed in the urogenital sinus. The division into a ventral urethra and

a dorsal vagina may entirely or partially be suppressed. As a result the sinus urogenitalis may develop into a urethra whereas the distal part of the vagina with its external orifice may be absent.

The uteri communicate with the proximal part of the vagina that develops from the sinus cord. This part lacks as a rule an orifice and in the adult the uteri, unable to discharge their contents may expand to monstrous or even fatal volumes. Some authors*) mention a communication of the vagina with the urethra.

5. The clitoris may assume the character of a penis. A normal urethra perforates the clitoris and it has its orifice at its top.

In intersexes penile tissue and also an os priapi may develop. Moreover the so-called plica balano-praeputialis may bear a dorsal split that cleaves the clitoris, which in the normal female remains a circular fold. A glans may evaginate.

Some authors*) have recorded other changes: a kind of hypospadia clitorialis with the urethra opening into the fissure on the dorsal side. This is not only observed after injection of androgens but also after that of oestrogens and progestational preparations: an indication that the influence is not specific but that it consist in an arrest of the normal development.

6. Sometimes an arrest of the nipple development has been mentioned and various investigators have reported abnormalities of the skeleton, skin, hair, salivary glands etc., but these changes seem of minor importance. In some respects the results of my own experiments deviate from those above enumerated.

TECHNIQUE

For the experiments white mice of our own breed were used. The pregnant female was subcutaneously injected with testosterone propionate in oil. It appeared that in order to

*) See Raynaud, A., pag. 112—162.

Table 4.

Date	Dosis	Days before part.	No. of litter mates	Alive or dead	Type of ♀ offspring	Age at autopsy in days
			♀	♂		
6.9.45	50 γ dd.	4	1	3 alive	vaginal	90
6.9.45	50 γ dd.	1	3	2 alive	vaginal	60, 60, 90
6.9.45	100 γ dd.	2	2	3 alive	vaginal	90, 90
21.9.45	50 γ dd.	7	3	4 2 ♂ dead	urethral	90, 90, 90
21.9.45	50 γ dd.	4	4	1 1 ♂ dead after 2 days	vaginal	50, 60, 60, 90
21.9.45	50 γ dd.	7	4	1 1 ♂ dead after 1 day	urethral	60, 60, 90, 90
2.10.45	50 γ dd.	3	1	0 alive	urethral	50, 60, 70, 90
16.10.45	100 γ dd.	5	5	2 alive	—	—
1.10.45	50 γ dd.	4	—	1 ♀ dead after 4 days	urethral	50, 60, 70, 90, 90
1.10.45	50 γ dd.	5	—	2 alive	vaginal	70, 90
1.10.45	50 γ dd.	6	—	— fetus born dead and unrecognizable by cannibalism	—	—
29.10.45	50 γ dd.	2	3	2 alive	vaginal	40, 90, 90
29.10.45	50 γ dd.	3	1	2 alive	vaginal	90
29.10.45	50 γ dd.	2	3	4 alive	vaginal	50, 60, 70
29.10.45	50 γ dd.	4	—	— abortion of dead fetus	urethral	60, 90
29.10.45	100 γ dd.	4	2	4 alive	—	—
29.10.45	100 γ dd.	3	—	— abortion of dead fetus	—	—
29.10.45	100 γ dd.	6	—	— abortion of dead fetus	urethral	35, 35
29.10.45	50 γ dd.	4	2	4 alive	urethral	50, 50, 50, 90
26.10.45	50 γ dd.	4	4	1 alive	urethral	120, 120
26.10.45	100 γ dd.	4	2	2 alive	vaginal	25, 35, 40, 45,
27.10.45	50 γ dd.	3	6	1 alive	vaginal	50, 60
26.10.45	100 γ dd.	3	2	3 alive	vaginal	35, 35
26.10.45	100 γ dd.	3	2	2 alive	urethral	50
20.11.45	100 γ dd.	3	3	1 2 ♀ and 1 ♂ dead	—	—
16.11.45	1×1000 γ	6	3	— resorption of fetus	—	—
20.11.45	1×500 γ	?	—	— abortion of fetus	—	—
20.11.45	1×500 γ	7	—	— mother dead 7 days after injection	urethral	35, 35, 90
20.11.45	1×500 γ	—	—	—	vaginal	120, 120, 120
19.11.45	1×500 γ	10	3	2 alive	urethral	120, 120
19.11.45	50 γ dd.	5	3	1 alive	urethral	90, 120, 120
19.11.45	50 γ dd.	4	2	4 alive	—	—
19.11.45	50 γ dd.	6	3	2 alive	—	—

Influence of testosterone propionate into the mother on fetal viability and female genital development of mice. (dd. = daily dose).

obtain living young the injection had to be administered after the seventh day of the pregnancy and that the doses had to be less than 100 μ per day; the best results were obtained when the injections were given in the three to five last days before delivery. The administration of higher doses or of injections at earlier stages of pregnancy did, in contradiction to other statements in the literature, in our experiments hardly afford result as the animals in most instances did not produce a living litter.

Even with our cautious treatment failure was not excluded: the mice sometimes miscarried or there was partus serotinus with the delivery of macerated foetus or no foetus was delivered at all, when the embryos disintegrated in the uterus. Other animals that were injected with doses of the same strength, however, bore viable youngs. (Table 1). The highest total amounts that were successfully administered varied between 350 and 500 μ ; they are low in comparison with the amounts with which other authors were able to obtain results, for the latter mention doses between 5 and 10 mg., and occasionally even higher amounts were used.

RESULTS

1. *Macroscopically visible abnormalities in the structure of the urogenital system of intersexual mice of female origin.*

New-born animals have not been autopsied. Our data pertain to animals of 35 to 120 days of age.

Ovary, tube and uterus are macroscopically normal (for microscopic details cf. II). Rests of the Wolffian duct were never met with, but this is not surprising since the treatment began, as a rule, after the physiological regression of these ducts.

The abnormalities proved to be confined to:

- a. the sinus urogenitalis and
- b. the clitoris.

ad a. The abnormalities observed in the sinus urogenitalis are not all of the same kind. Two main types may be distinguished,

each of them with variations but there is no gradual change from the normal condition via one type to the other.

The orifice of the urethra, which in normal females is found at the top of the clitoris (Fig. 1 A) usually in a short vertical



Fig. 1 A.

Urogenital system of normal adult female mouse.

groove, shifts under the influence of small doses of androgen (2—3 times 50 γ) to the base of the clitoris or sometimes even a little way up into the vagina. For this type, in which urethra and vagina have a common orifice through the normal orifice of the vagina we will use the name *vaginal type* (Fig. 1 B). Doses of the same strength, however, can produce an entirely

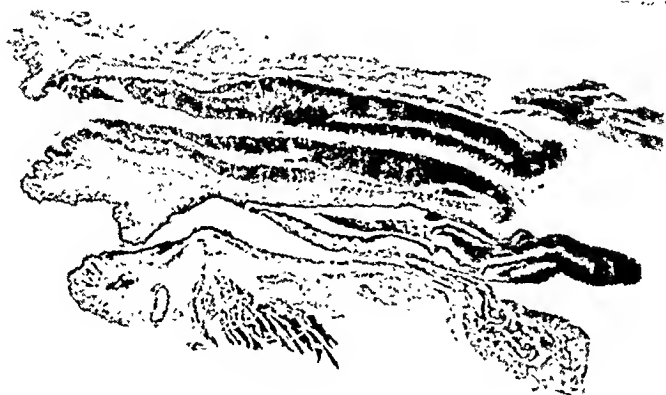


Fig. 1 B.

Urogenital system of adult female mouse with experimental intersexuality (vaginal type).

different type of abnormality, although the latter is more easily obtained by longer treatment or a stronger dose (see table 1). In this case the urethra opens either at the dorsal side of the clitoris or on the top of it, but the orifice of the vagina and, in fact, the whole distal part of the latter is absent. The proximal part of the vagina may assume funnel

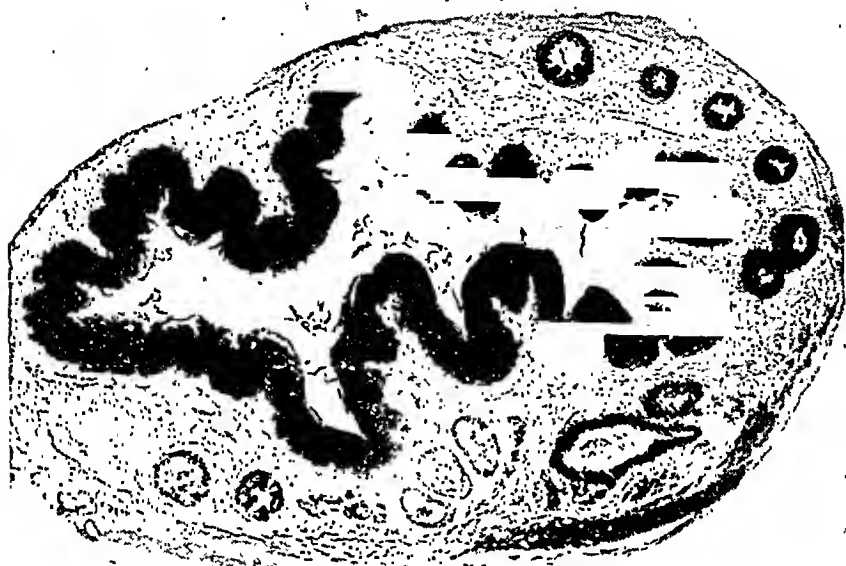


Fig. 2.

Paravaginal ducts in mouse with vaginal type of intersexuality.

shape and discharge above the floor of the pelvis into the urethra. The common outlet is then through the urethra, whose external position is normal. We call this *the urethral type*.

By the administration of androgen the normal course of development of the sinus urogenitalis is disturbed, but the ensuing difficulties are solved in such a way that the animal remains alive. It is noteworthy that also in cases of spontaneous humoral (hormonal) intersexuality in women (in affections of the adrenal cortex) a vaginal and a urethral type are distinguishable.

ad b. The clitoris also undergoes changes, but there is some

degree of correlation with the types considered in the preceding paragraph. In the vaginal type the clitoris is broad and of a sturdy build, and it is distinctly larger than normal. On the dorsal side a cleaving takes place, and the urethra finds its outlet in this cleft at the base of the clitoris. The cleft itself has on each side a deep red swelling of the mucosa. Laterally these swellings pass into the skin, cranially into the mucosa of the vagina. When the urethra opens into the vagina, the cleaving is often slight, but the clitoris is nevertheless broad and at the dorsal side showing the mucosal swelling.

In the urethral type the clitoris is, as a rule, normally developed, although sometimes a slight cleft and a swelling of the mucosa may be noted on the dorsal side. Sometimes the urethra opens exactly at the top of the clitoris and then the latter is externally of entirely normal shape and not enlarged.

II. *Microscopical aspect of the abnormalities in the structure of sinus urogenitalis and clitoris.*

A. *Sinus urogenitalis.*

1. *Vaginal type.*

In the vaginal type, where the urethra opens into the vagina, the ventral wall of the common outlet is covered with urethral epithelium. This epithelium also extends some way on the dorsal face of the clitoris, where it forms the mucosa swelling to which reference already has been made. The dorsal wall of the common outlet is covered with vaginal epithelium.

The clitoris is not perforated by the urethra, and is entirely filled with adipose tissue. Occasionally a rudiment of the os priapi is found. The plica balano-praeputialis is not cleft. Around the vagina a peculiar differentiation of tubuli is noted. (v. infra).

2. *Urethral type.*

Fig. 2 represents a section through the vagina at the height of the neck of the bladder. In the connective tissue between

vagina and urethra ganglia are found. In the connective tissue round the vagina small groups of ganglion cells are more frequent than normally. The connective tissue round these groups of nerve cells is often seen to be denser than usual, and epitheloid elements are present.

A few sections lower a group of epithelial cells is found to form a cord extending in a distal direction in which as a rule, a lumen is formed. The number of these tubuli varies and it may be considerable. The first ones appear, as a rule, at the height of the bladder neck, 5 to 10 on each side. They sometimes communicate. Further down new tubuli arise, soon closing to a ring round the vagina. Where the vagina opens into the urethra, tubules are separated into two groups, one ventrolaterally from the urethra, the other dorsolaterally, from the vagina. The tubuli round the urethra have sometimes openings in the latter, but more often they communicate with the glandular tissue situated ventrolaterally from the distal end of the urethra (peri-urethral glandular tissue). The tubuli round the vagina usually open into the latter, but they may also end blindly. The epithelium of these tubuli always resembles that of the vagina, no matter whether they open into the vagina or into the urethra. Their lumen is, as a rule, rather narrow, but sometimes it may exceed that of the vagina. The contents vary: they may consist of mucous detritus with leucocytes and epithelial rests, but the epithelium may also be cornified, and then the lumen contains cornified epithelial pearls. Mucification and cornification may be present in adjacent tubuli, and even in different parts of a single tubulus. Horn masses may also be present in cases in which the vagina does not show the slightest trace of cornification, but on the contrary is covered with mucoid epithelium.

Fig. 3 is a section at the height of the opening of the vagina into the urethra. The presence of numerous folds in the epithelium of these tubuli is noteworthy, the common outlet of the urethra and the vagina has at the dorsal side epithelium of the vaginal type, and at the ventral side epithelium of the urethral type. Distally the wall is on all sides covered by urethral epithelium.



Fig. 3.

Opening of vagina in urethra in mouse with urethral type of intersexuality.

The peri-urethral glands are always well developed. (Fig. 3). In the distal part of the urethra its lumen assumes the shape of an anchor. This is the region of the sinus urethralis.

In the blades of the anchor are the orifices of the excretory ducts of Cowper's glands. These glands themselves may show a marked distension, even where a satisfactory connection with the urethra seems to be present.

B. Clitoris.

In the clitoris we found ventrally from the urethra a stiff connective tissue provided with a few venous lacunae. This is to be interpreted as cavernous tissue. In the urethral types an os priapi is always present, in the vaginal type often. The plica balano-praeputialis is normal, often strongly plicated, but never split. The praeputial glands are large and often markedly distended.

The most striking peculiarity of mouse intersexes is the development of the numerous tubuli round the vagina with their different kind of epithelium. They show that there is a strong tendency to form evaginations; this manifests itself also in the numerous folds observed in the epithelium. The formation of these tubuli replaces the development of a prostate as described by other authors and as observed by ourselves in experiments with rats. At first view one would be inclined to regard the tubuli as homologous to the prostate tissue, but the circumstance that the tubuli are also found at the dorsal side of the vagina as well as their extension in distal direction, makes one rather sceptic with regard to the correctness of this interpretation.

The nipples in our animals, no matter of what type they are, were always well developed. There is no sign of a disturbed development, as has been mentioned in the literature.

III. *Functional condition of the genitals in experimentally induced intersexuality.*

Adult mice and rats with experimentally induced intersexuality have comparatively seldom been studied. Some authors*) describe premature oestrus that may seem to be persistent. This is in agreement with the condition of the ovaries in which an early and strong follicle development is observed. Corpora lutea are only exceptionally reported in such ovaries.

In our investigation, 67 to 120 days old animals have been examined. Our description applies to animals of the urethral type as well as to animals of the vaginal type.

To begin with, the relative frequency of the nuclei in corpora lutea of the cycle as well as in corpora lutea of pregnancy was determined in normal mice. In our measuring apparatus the number of nuclei per unit of volume (a constant surface area multiplied with the constant thickness of the section) appeared to be in corpora

*) See Raynaud, A., pag. 229—234.

lutea of the cycle 40—44, and in corpora lutea of pregnancy 19—21, i.e. about half as much. This means that the volume of a single cell in a corpus luteum of pregnancy is about twice the volume of a cell of a corpus luteum of the cycle.

C. Ovary.

In three of the nine animals, sectioned at the age of 35 days, corpora lutea were found. The follicle development in



Fig. 4.

Corpora lutea of pregnancy type in ovary of mouse with intersexuality.

the ovaries of all animals was good and old structures were frequent. The relative frequency (N_R) of the nuclei in the corpora was 42. The occurrence of corpora in 35 days old animals can be considered as abnormal, as in normal mice of our strain the ovary does not contain corpora lutea before the animal is 45—50 days old.

In two of the three animals of 40—50 days corpora were found with a $N_R = 40$.

In the ovaries of 50 days old mice there were corpora lutea of the cyclic type (Fig. 4). However, in one animal the ovary contained corpora lutea of pregnancy type with a N_R count of 21.

The same phenomenon was observed in animals 60, 70, 90 and 120 days old (see Table 1).

In some animals each ovary contained more than 20 corp. lutea of pregnancy, all of them showing a N_R of 20—21. Such ovaries looked like one that had been artificially luteinized with a gonadotrophin (Fig. 4). The vagina of such animals showed



Fig. 5.

Vagina with rests of mucification of the epithelium.

in some instances a distinct mucification or at least a corresponding intermediate stage between cornification and mucification, that was never met with in normal mice (Fig. 5).

The changes described above were not always met with, the frequency of corpora lutea of pregnancy and mucification of the vagina can be seen in Table 2. In the other mice the vagina showed the oestrous appearance and the ovary contained no large corpora lutea resembling corpora lutea of pregnancy. Sometimes, however, old structures, suggesting that such corpora may have been present, were found.

It seems almost as if the animals with the large corpora lutea possess a three-phasic cycle. The diphasic cycle of the normal mouse seems to be supplemented by a progestational phase, as is normal in anthropoid apes and in women. It is

difficult to ascertain whether this cycle is regular and how long it lasts. It is in this connection of importance to note that the mammary glands were always undeveloped though some of the animals were unquestionably pseudopregnant at the time of autopsy. This may indicate that the progestational phase of the cycle cannot have lasted long.

Table 2.

Age	Type of animals	No.	Number of animals with c. l. grav.	Number of animals with mucified vagina
120	vaginal	3	2	1
	urethral	6	3	2
90	vaginal	9	3	1
	urethral	12	4	2
70	vaginal	2	1	0
	urethral	2	0	0
60	vaginal	6	2	1
	urethral	5	0	0
50	vaginal	3	0	0
	urethral	6	1	0

Progestational phase of cycle in intersexuality.

D. Adrenal cortex.

Raynaud is, as far as we know, the only author who has paid some attention to the adrenal cortex of intersexes. He finds hypertrophy of the X-zone. The adrenal cortex of the mouse consists of three zones: an outer one, the zona glomerulosa; a middle one, the zona fasciculata and an inner one, the zona reticularis. The last named zone is also known as X-zone or as androgenous zone. The X-zone is present in young animals only; in the male it disappears after 4 weeks, in the female after 2—3 months. The process is accelerated by pregnancy. After the disappearance of the X-zone the medulla is surrounded by a thin membrane consisting of connective tissue in which small groups of cells remain visible.

The only constant reaction of the X-zone in the young mouse, which may be regarded as the equivalent of the X-zone of the adrenal cortex in the human embryo, is its disappearance after injection of the animal with androgens.

In the mouse the X-zone is believed by some authors to consist of a special kind of cells and to be fundamentally dif-



Fig. 6.

Corpora lutea of the cycle ovary of normal mouse.

ferent from the other zones. Others disagree with this view.

An examination of the adrenal glands of our test animals show that no abnormalities worth mentioning were present until they have reached an age of 6 to 8 weeks. The X-zone was, just as in normal animals of this age, strongly developed. When the animals were 8 to 12 weeks old, the X-zone began to exhibit differences: in the normal animals it was degenerated (Fig. 7 A), but in the test animals it was maintained and it even continued its growth. The weight of the adrenal glands increased in the test animals in this way to 6 or 8 mg., whereas in the normal ones it remained at 4—5 mg.

Occasionally large adrenal glands were met with, weighing 10 to 15 mg. Their X-zone proved to be strongly hyperplastic (Fig. 7 B). Sometimes, they have shown an extraordinary

change: the cells of the X-zone were swollen and vacuolized, the zone appearing as adipose tissue. This »adipose-zone« is seen in the section to surround the medulla as a wide ring.



Fig. 7 A.

Adrenal gland of normal adult female mouse.

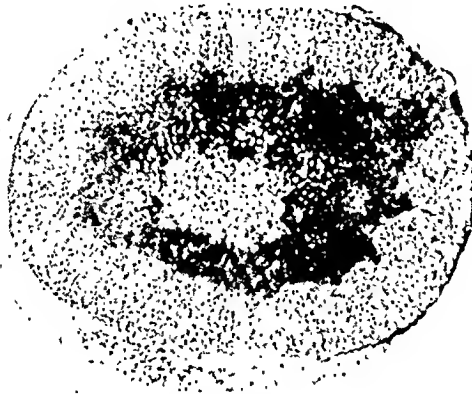


Fig. 7 B.

Adrenal gland with hypertrophic X-zone of adult female mouse with intersexuality.

Its histiogenesis was revealed by the presence of small groups of non adipose cells scattered through the tissue (Fig. 7 C).

The peculiar behaviour of the X-zone and particularly the fact that the fatty degeneration remains entirely confined to this zone, favours the view of a separate character assigned to this zone, though fatty change may also mark the ultrafinal stage in the life of aging cells.

DISCUSSION

Relatively minute amounts of androgen (testosterone propionate) injected in pregnant mice during the last days of the pregnancy period, induced in our experiments anomalies in the structure of the urogenital system. These anomalies appeared to be confined to the region of the sinus urogenitalis and the external genital organs. Two types of intersexes could be distinguished: a vaginal and a urethral type.

No prostate tissue was found, but the vagina and urethra was surrounded by a system of tubules surmounted by nerve

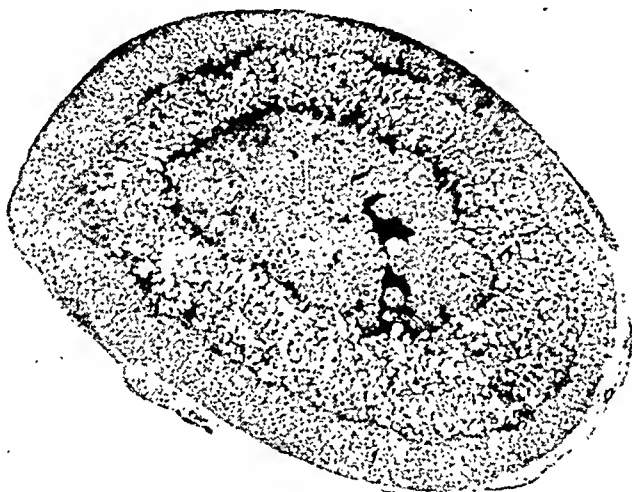


Fig. 7 C.

Fatty degeneration of hypertrophic X-zone in adult female mouse with intersexuality.

cells. The ovary kept its normal structure, but maturation was accelerated to a kind of *pubertas praecox*. In the absence of true pregnancy ovary as well as uterus and vagina were often found in a progestational condition; in a kind of pseudo-pregnancy.

The adrenal cortex also underwent remarkable changes, which might indicate the presence of a strong stimulus.

Other authors have noticed only the early development of the follicles (*pubertas praecox*) and Raynaud pointed out a strong development of the X-zone in the adrenal cortex.

How are these anomalies brought about? As the animals did not receive a special treatment after their birth, the cause of the postfetal abnormalities should be intrinsic. Anomalies of the urogenital sinus and of the clitoris or of the clitoris alone suggest themselves as possible causal factors.

It is known that the region of the sinus urogenitalis and the clitoris is a receptor area from where stimuli are transmitted to the endocrine system (during the coitus or incident to mechanical or electrical stimulation). In this way a change is induced in the hypophysis (gonadotrophic hyperpituitarism) which makes itself felt in the ovary. The latter responds by precipitated follicle maturation and by the development of corpora lutea of pregnancy; in this way a so-called pseudo-pregnancy is induced.

Stimulation of the nipples, e. g. by sucking young, induces in the hypophysis another change (lactotrophic hyperpituitarism) by which lactation is started and maintained. In view of the important part played by serial relations of the kind: stimulation of receptor field — hypophysis activity — activity of the dependent organs, it is conceivable that the abnormalities in the structure of a receptor area (sinus urogenitalis and clitoris) in the intersexes among our mice has produced abnormal stimuli which eventually affect the gonad and the adrenal gland: the gonad reacting with a premature development of follicles and corpora lutea and the adrenal gland with hypertrophy of the X-zone, and, eventually with its fatty degeneration.

SUMMARY

Prenatal treatment of mice with low doses of testosterone propionate (150—500 γ) resulted in abnormalities (vaginal- and urethral type) of the distal part of the female urogenital system. Paravaginal ducts were found. In the ovaries of adult animals corpora lutea of pregnancy type, associated

with mucification of the vagina, and sometimes a remarkable fatty degeneration in the hyperplastic inner (X-)zone of the adrenal cortex were demonstrable.

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COMPARATIVE STUDIES OF THE HEIGHT OF THYROID CELLS IN DIFFERENT FIXATIONS

BY

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Every histologist must realize, that histological methods are, to a great extent, imperfect and in general give only an incomplete picture of the exact relationship within the cell and tissue.

Not only are there greatly varying changes caused by the various fixing materials themselves, but the necessary fixation treatment of the living tissues causes far reaching changes in the vital relationships. The above mentioned statements may be thought to be so obvious to every histologist as to be superfluous. However, a study of literature on the same subject reveals that these »obvious« observations are not always considered, so that we believe it necessary to at least draw attention to them.

Fixation and further treatment of various tissues causes swelling and shrinkage in various degrees, depending both on the treatment and the tissues.

In studies of the thyroid gland's activity, one has generally used the follicle cells' height as a true measure of gland function. It is of course to be admitted, that in all similar cases, it is of the greatest importance that the treatment of various preparations will be as homogeneous as possible, otherwise the results may be misinterpreted. It is of the greatest importance that each investigator uses the same methods, or at least under-

stands that different treatments can yield different results. Comparisons of results from different investigators must be analysed with the above statements in mind.

In general, total shrinkage with fixation and paraffin embedding is approximately 10—30 per cent, depending on the nature of specimen, fixation and further treatment. It is interesting to note that osmium tetroxid seems to cause a relatively insignificant shrinkage. *Hursh* (1939) found a shrinkage of 10.1 ± 0.16 per cent in isolated nerve fibres. He even showed that when the whole nerve shrunk by 23.6 ± 1.2 per cent, the connective tissue shrank more than the nerve fibres. *Rexed & Swensson* (1941) compared nerves fixed with osmium with nerves fixed and stained by Alzheimer-Mann method modified by *Häggqvist* (1936). They showed that the latter method produced 25 per cent shrinkage. Later *Rexed* (1944) showed that in nerves treated by both above mentioned methods, the nerve fibres' diameter shrank 24.4 ± 3.9 per cent less in fixation and staining by Alzheimer-Mann than by osmium treatment. Other methods such as Weigert's, showed that they too had the same shrinkage effects as Alzheimer-Mann. Of even greater interest is the author's discovery that the diameter of nerve fibres after 24 hours fixation in 10 per cent formaldehyde is the same as in original nerve tissue. In 4 per cent formaldehyde, however, there is a definite swelling effect.

According to various investigators (lit. see *Rexed*, 1944) the fixation in formalin and various formaldehyde concentrations causes different changes in volume. Thus, formaldehyde solutions 5 per cent, causes swelling, 10 per cent solutions will not cause any changes. It must be mentioned that *Patten & Philport* (1921), investigating fixation and embedding changes on length of pig embryos, found that 4 per cent formaldehyde caused 5 per cent swelling. Shrinkage when preparations were ready for paraffin embedding rose to approximately 20.5 per cent. *Block* (1948) states that length shrinkage of ovaries fixed in 4 per cent formaldehyde was 12.7 per cent and volume shrinkage about 33.4 per cent.

With reference to thyroid cell and gland cell relationships

in different fixations, there does not exist, as far as we know, any work that previous to this paper has treated more deeply in histological manner, this fundamental problem of activity measurements. A study of the literature which attempts through measurement of thyroid cells' height to estimate the gland's activity, reveals that the gland has been fixed in various fixatives such as formaldehyde, Susa, Zenker's solution, Bouin's solution (lit. see *Borell & Holmgren*, 1943, *Borell*, 1945). Of interest are *Wilckes'* (1934—36) investigations of the thyroid gland's histological picture by fixation with Susa and formaldehyde. The author has used thyroid material from untreated guinea-pigs, some from pigs treated with thyrotrophin. The gland's activity has been judged according to Heyl's and Laquer's scheme. Wilcke found in most cases a higher epithelium in Susa fixed thyroid glands than in formaldehyde treated material, which shrank instead. Wilcke's formol is a 10 per cent solution of the usual unneutralized commercial formalin.

MATERIALS AND METHODS

In the studies being described we have used male guinea pigs weighing approximately 300 grams. After the animal was killed, the thyroid gland was removed and both lobes were divided into a number of equal pieces, which were fixed in the following solutions: 4, 10 and 20 per cent formaldehyde for 19 hours, Susa (24 hours), Bouin (18 hours) and Carnoy (4 hours). After fixing the specimens were dehydrated and embedded in paraffin in similar manner. Then the specimens were sliced into 5 μ thick slices and stained with iron-alum-haematoxylin (*Häggqvist*).

Half of the material came from untreated animals, the other half came from guinea-pigs who had received 10 GPU thyrotrophin every day for 5 days.

Cell height was measured with the assistance of an ocular-micrometer. The method is described more in detail by *Borell & Holmgren* (1943) and *Borell* (1945).

Table 4.

Follicle cell height — in microns.

Animal number	Formaldehyde			Susa	Bouin	Carnoy
	4 per cent	10 per cent	20 per cent			
949	$7,7 \pm 0,10$ $\sigma = \pm 0,52$	$7,2 \pm 0,11$ $\sigma = \pm 0,55$	$7,1 \pm 0,14$ $\sigma = \pm 0,63$	$10,2 \pm 0,11$ $\sigma = \pm 0,56$	$11,0 \pm 0,21$ $\sigma = \pm 1,03$	$7,3 \pm 0,14$ $\sigma = \pm 0,72$
950	$7,2 \pm 0,17$ $\sigma = \pm 0,86$	$7,7 \pm 0,03$ $\sigma = \pm 0,17$	$6,3 \pm 0,17$ $\sigma = \pm 0,89$	$10,0 \pm 0,11$ $\sigma = \pm 0,57$	$9,9 \pm 0,33$ $\sigma = \pm 1,63$	$8,1 \pm 0,14$ $\sigma = \pm 0,14$
951	$6,5 \pm 0,21$ $\sigma = \pm 1,23$	$7,7 \pm 0,10$ $\sigma = \pm 0,49$	$6,5 \pm 0,29$ $\sigma = \pm 1,45$	$10,6 \pm 0,13$ $\sigma = \pm 0,66$	$10,9 \pm 0,33$ $\sigma = \pm 1,64$	$7,1 \pm 0,17$ $\sigma = \pm 0,87$
952	$6,8 \pm 0,25$ $\sigma = \pm 1,23$	$7,3 \pm 0,16$ $\sigma = \pm 0,79$	$6,6 \pm 0,23$ $\sigma = \pm 1,13$	$10,0 \pm 0,15$ $\sigma = \pm 0,73$	$10,6 \pm 0,40$ $\sigma = \pm 1,98$	$6,7 \pm 0,25$ $\sigma = \pm 1,25$
953	$7,2 \pm 0,27$ $\sigma = \pm 1,33$	$7,6 \pm 0,23$ $\sigma = \pm 1,15$	$6,9 \pm 0,22$ $\sigma = \pm 1,09$	$10,4 \pm 0,11$ $\sigma = \pm 0,55$	$10,8 \pm 0,38$ $\sigma = \pm 1,91$	$7,4 \pm 0,24$ $\sigma = \pm 1,19$
Mean	7,1	7,5	6,7	10,1	10,6	7,3

973	$10,1 \pm 0,34$ $\sigma = 1,66$	$12,1 \pm 0,17$ $\sigma = 0,85$	$11,6 \pm 0,22$ $\sigma = 1,08$	$12,4 \pm 0,15$ $\sigma = 0,77$	$12,2 \pm 0,35$ $\sigma = 1,73$	$10,4 \pm 0,26$ $\sigma = 1,20$
974	$10,6 \pm 0,33$ $\sigma = 1,66$	$11,1 \pm 0,30$ $\sigma = \pm 1,52$	$11,2 \pm 0,32$ $\sigma = 1,60$	$12,4 \pm 0,14$ $\sigma = 0,72$	$11,8 \pm 0,39$ $\sigma = 1,94$	$10,5 \pm 0,27$ $\sigma = 1,35$
975	$11,5 \pm 0,23$ $\sigma = 1,50$	$11,8 \pm 0,25$ $\sigma = 1,24$	$11,7 \pm 0,31$ $\sigma = \pm 1,55$	$12,6 \pm 0,17$ $\sigma = \pm 0,83$	$12,9 \pm 0,35$ $\sigma = 1,73$	$11,9 \pm 0,27$ $\sigma = \pm 1,37$
975	$11,3 \pm 0,22$ $\sigma = 1,44$	$11,6 \pm 0,23$ $\sigma = 1,15$	$11,3 \pm 0,32$ $\sigma = \pm 1,61$	$12,6 \pm 0,12$ $\sigma = \pm 0,51$	$12,3 \pm 0,37$ $\sigma = 1,85$	$10,6 \pm 0,38$ $\sigma = 1,74$
977	$10,8 \pm 0,32$ $\sigma = 1,62$	$11,1 \pm 0,25$ $\sigma = \pm 1,23$	$11,9 \pm 0,29$ $\sigma = 1,44$	$12,4 \pm 0,15$ $\sigma = 0,77$	$12,4 \pm 0,40$ $\sigma = 1,98$	$10,6 \pm 0,35$ $\sigma = 1,74$
Mean	10,9	11,5	11,3	12,5	12,3	10,8

Animals no. 949—953 untreated.

Animals no. 973—977 treated with thyrotrophin (Ambinon, Organon).

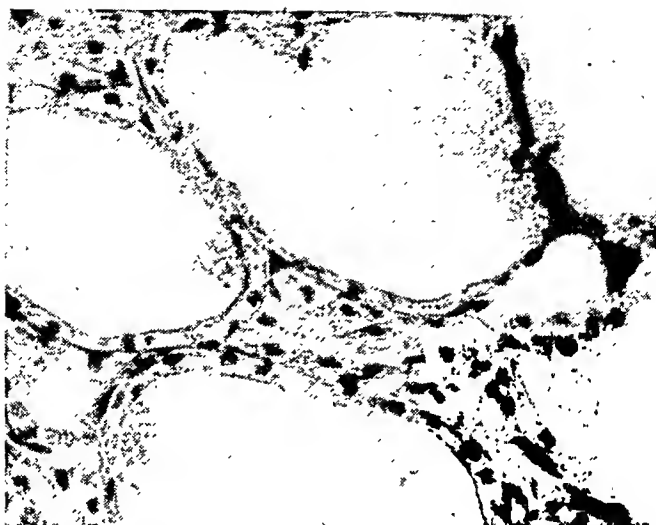
RESULTS

Table 1 gives the values for the cell heights of the glands fixed in various manner. The first five animals (949—953) are normal, the following five (973—977) were treated with thyrotrophin. This was done so as to ascertain if changes in cell height of active glands in various fixing solutions are similar to changes in normal glands.

From the results it can be seen that cell heights are the same, to a great degree when the gland is fixed in different formaldehyde concentrations. It is of interest to note that cell heights in certain cases except one (No. 974) are smallest in fixing with 20 per cent formaldehyde. The difference between the values of cell heights in normal thyroid glands fixed in 10 and 20 per cent formaldehyde is $0.8\ \mu$. The cell height differences for thyrotrophin treated animals is $0.2\ \mu$.

Comparison between values for cell heights for glands fixed in Susa and Bouin and those already described, however, reveals greater differences. The difference between the Susa value for normal animals and the corresponding value for glands fixed in 10 per cent formaldehyde is $2.5\ \mu$. Corresponding difference between Bouin and formaldehyde fixed glands is $3.1\ \mu$. The differences of cell heights in the thyrotrophin series are not quite so large. In the first case the difference is $1.0\ \mu$, in the other case $0.8\ \mu$. It seems that here the different fixing solutions do not affect the cell heights as much as in normal cases. The cell height of glands fixed in Carnoy agrees best with the value in a 4 per cent formaldehyde fixed thyroid specimen. The reason that these two fixing solutions give one and the same result is that in the 4 per cent formaldehyde the partly hardened tissue shrinks considerably in dehydration, which shrinkage also occurs when the fresh gland is fixed in the strongly alcoholic Carnoy. The differences between the values for cell height in Carnoy thyroid glands and the glands fixed in Susa and Bouin, both for normal animals and animals treated with hormones, are strikingly large.

If one finally compares the values of cell heights for Susa and Bouin fixed thyroid glands, no large difference can be



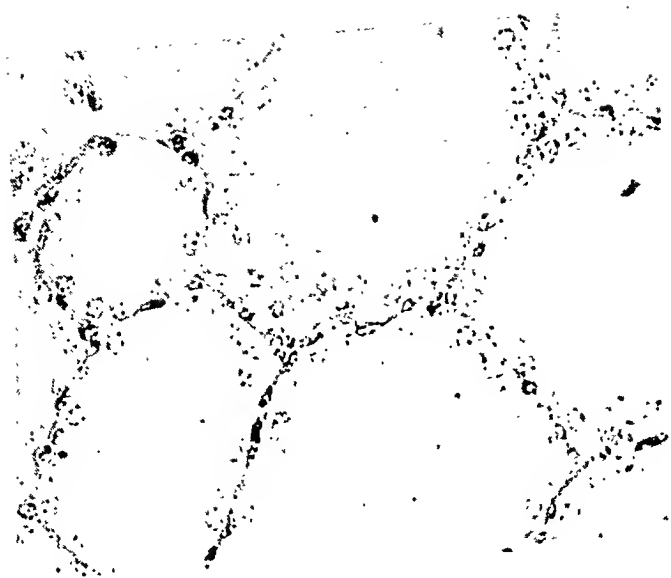
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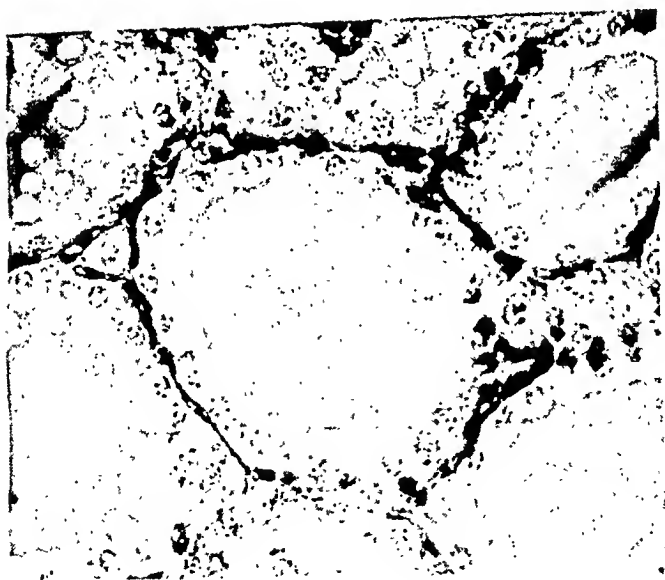
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Fig. 1.

- a) Thyroid fixed in 4 per cent formaldehyde. Low epithelium height (6.5μ). Nucleus and cytoplasm greatly destroyed. Colloid vanished, Animal 951.
- b) Thyroid fixed as in a). Animal treated with thyrotrophin. Epithelium height 11.5μ . Colloid as in a). Animal 975.



a



b

Fig. 2.

a) Thyroid fixed in Bouin. Epithelium height $9.9\ \mu$. Nucleus and cytoplasm well fixed, colloid also well fixed.

b) Same thyroid as in 2 a) fixed in Susa. Epithelium height $10.0\ \mu$. Also in this case good fixing of nucleus and cytoplasm. Animal 950.

found. It seems necessary to point out, however, that the margin of error for Susa fixed thyroid glands is considerably less than for Bouin fixed glands. This is so not only because the cell heights are more even in the individual gland in the first case, but also because it is easier to see the cell boundaries in Susa fixed preparations than in Bouin fixed cells, thus it is easier to get more accurate measurements.

The different fixing solutions affect not only the cell heights of the cells. In formaldehyde colloid is, in general, badly fixed, especially in 4 per cent solution (fig. 1). In these preparations one finds none, or only isolated remains of colloid in the follicles. In the higher concentration the colloid is better fixed. The cytoplasm in the follicle cells is very badly preserved in formaldehyde solution, especially in the 4 per cent solution (fig. 1). In most cells, the cytoplasm is seen only as a thick, wormlike, uneven net. The nucleus is seen as a dark, compact body with very little chromatin. In general, it appears that formaldehyde in low concentrations is a very unsatisfactory fixing medium. In higher concentration of formaldehyde, the cytoplasm and the nucleus is better preserved. Cell and nuclear membrane, as well as chromatin can be readily differentiated.

In Susa and Bouin the colloid is much better preserved than in formaldehyde fixed preparations (fig. 2). Follicle cells are well defined, the cytoplasm shows an even net-like structure. The cells do not present an even surface in the follicle lumen but present more or less cupole-like surface against the lumen. Especially is this noticeable in thyroid cells from hormone treated animals (fig. 3). The nuclei with well defined nuclear membrane and marked chromatin nets, can be seen.

The colloid is well fixed in Carnoy and especially in the follicles on the periphery the colloid fills the follicle lumen. Cell boundaries are easily seen and the cytoplasm has a net-like structure. The nucleus has a marked membrane and a very clear chromatin net.

Of all the fixing materials tested, the last three give the best cytological pictures. Carnoy fixes quickly but unevenly,

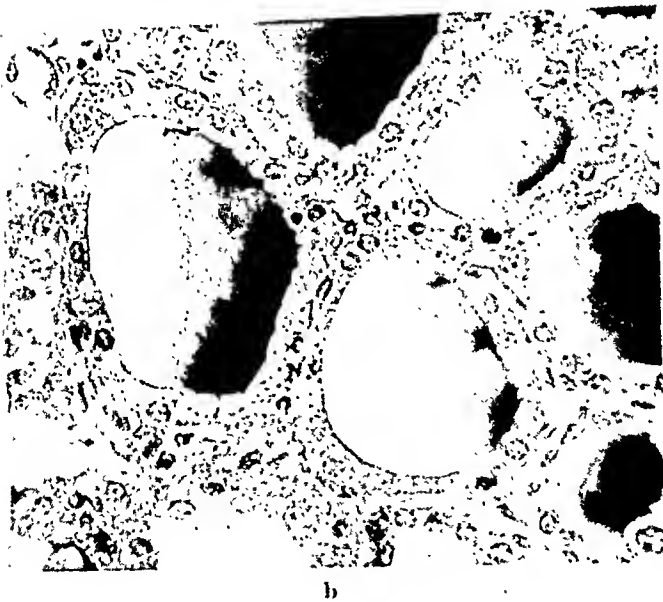
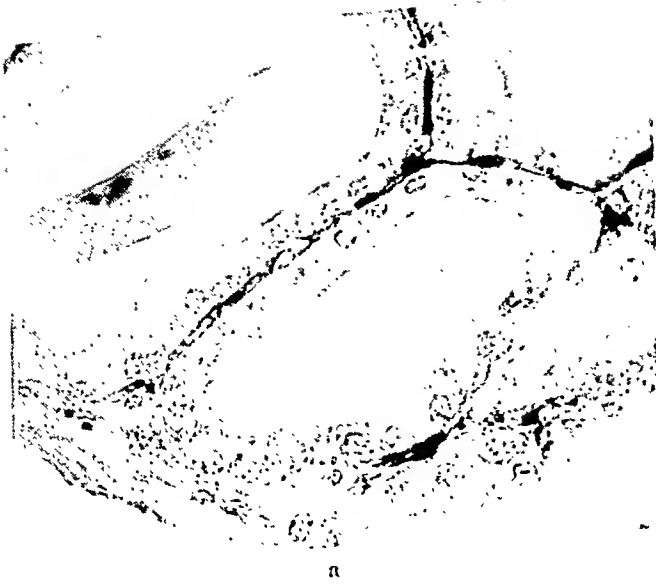


Fig. 3.

- a) Thyroid fixed in Bouin. Cells well preserved. Colloid relatively well preserved. Epithelium height 11.8μ .
- b) Same thyroid fixed in Carnoy. Good fixing. Cell height 10.5μ . Animal 974.

so that the outer parts of the preparation harden the best. Susa and Bouin give, instead evenner and surer results.

The fixation experiments have shown with extreme clarity, that the method of fixation has a great effect on the final form of the individual cell. The results have shown unquestionably that in all studies for the ascertaining of cell changes the same fixative must be used throughout. Even the time and later treatment must be the same. It is important that the cell boundaries and other details can be easily seen, in other words the fixing process must be as complete as possible. One must always remember that the microscopes' image is not the same as the true form. *Carleton* (1938) stated the above in the following words »In fact the fixed cell is to protoplasma what the embalmed body is to a living man — a more or less accurate imitation«.

In the thyroid studies we have made (*Borell & Holmgren*, 1943, *Borell*, 1945, *Borell & Holmgren*, 1948, *Holmgren & Naumann* — not published) we used Susa fixation for 24 hours to harden the glands. This we have done so as to keep a homogeneous material which could be used for comparison purposes.

Our studies have clearly shown the variations of cell height caused by different fixing methods. As cell heights are often used as a measure of thyrotrophin effectivity, it is stressed that various investigators use a uniform technique. As formaldehyde is a very poor fixative, cytologically speaking, we suggest that in the future Susa or Bouin will be used as fixing solution.

SUMMARY

The cell heights have been measured of guinea-pigs' thyroid glands in various fixing solutions with help of an ocular micrometer. The following results have been found:

- 1) In 4—20 per cent solutions of formaldehyde the cell height is practically the same, for normal glands and activated glands.

- 2) Susa and Bouin solutions give the highest values and both lie at the same height.

3) Carnoy fixing gives the same values as fixing in a 4 per cent formaldehyde solution.

4) Susa and Bouin give the best histological results. The preparations are uniform and well fixed.

5) The necessity of a uniform method in cell height measurement experiments is strongly indicated.

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THE INFLUENCE OF ESTERIFICATION UPON THE BIOLOGICAL ACTIVITY OF OESTRADIOL IN RATS AND MICE

BY

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The protracted influence of the esters of oestrogenic derivatives of phenanthrene was first demonstrated by *Butenandt* (1931) and *Butenandt & Störmer* (1932), who assumed that the cause was to be found in a slow saponification of the esterified forms in the blood. Afterwards, however, *Deanesly & Parkes* (1937) showed that in the case of intravenous injection there was no difference in the duration of the effect of the free and the esterified substances; and after the studies published by *Pedersen-Bjergaard* (1939), the explanation must be the different rate of resorption from the oil depots. Studies of a number of aliphatic esters of oestrone and oestradiol have proved that the protracted effect is proportional with the length of the acid chain, but that sometimes this protracted effect is achieved at the expense of the strength of the preparation (*David, de Jongh & Laqueur*, 1935, *Parkes*, 1937, *Deanesly & Parkes*, 1937, and *Miescher, Scholtz & Tschopp*, 1937).

In the present work the significance of the strength and the duration of effect of esterification on the substances was examined partly by means of the vaginal cornification test on

mice and rats, and partly by the muscle activity test on adult spayed rats. The question of resorption from oil depots was also gone into. Oil solutions of oestradiol, oestradiolmonobenzoate and oestradioldipropionate were employed.*)

EXPERIMENTAL.

1) *Determination of the effective dose.*

The oestrogenic activity of oestradiol, oestradiolmonobenzoate and oestradioldipropionate was determined by the vaginal cornification test on adult spayed mice (average weight 20 g.) and rats (average weight 200 g.), which were given one subcutaneous injection of an oil solution. The vaginal smears was examined from 50 to 100 hours later (with the small doses used a longer period of observation was unnecessary).

The quantity producing vaginal cornification in 50 per cent of the animals was selected as the unit or effective dose (E. D. 50). The result is shown in Table 1, from which it will be seen that the dose of O. D. P. must be four times that of O. M. B. for mice and twice as much O. D. P. as O. M. B. for rats; in the case of mice it requires 88 times as much free O and for rats 26 times as much free O as O. M. B. when the dose is applied in one oil injection.

Table 1.

Quantity of oestrogenic substance in oil inducing vaginal cornification in 50 per cent of the animals after one subcutaneous injection.

	μg oestradiol	μg oestradiolmonobenzoate	μg oestradioldipropionate
spayed mice	4.4	0.05	0.2
spayed rats	5.2	0.20	0.4

*) The abbreviations O, O. M. B. and O. D. P. are occasionally employed in the text.

2) *Duration of oestrus measured by vaginal cornification test.*

The results of the experiments with vaginal cornification as a test object are shown in Table 2. Twenty animals were employed for establishing each value. The deviation with which each determination was made is much greater than that involved in the values given in Table 1 and amounts to about 25 per cent.

Table 2.

Average number of days of vaginal cornification after administering the oestrogenic substance in one oil injection.

Substance in μg	Spayed mice				Spayed rats			
	Oestradiol			O. M. B. O. D. P.	Oestradiol			O. M. B. O. D. P.
	Free	benzoate	diprop.		Free	benzoate	diprop.	
	days	days	days		days	days	days	
0.4	0	6	9	1/1.5	0	2	5	1/2.5
4.0	0	12	17	1/1.4	0	3	8	1/2.7
40.0	5	22	30	1/1.4	3	6	17	1/2.8
400	12	34	43	1/1.3	4	13	36	1/2.8
4000	—	61	87	1/1.5	—	19	75	1/3.9

It will be seen from Table 2 that the esters have a considerable protracted effect compared with the free oestradiol. The relation between dose and duration of effect appears from Figs. 1 and 2, which shows that there is linear dependence between the logarithm of the dose and that of the duration of the effect as expressed in the number of days.

If with the help of Figs. 1 and 2 we find the doses of O. M. B. and O. D. P. that produce the same effect-duration of vaginal cornification in mice and rats respectively, we arrive at the values shown in Table 3.

It will be seen that the longer the effect is desired to be, the greater is the difference between the required doses of O. M. B. and O. D. P. for rats. For mice, however, the relation between O. D. P. and O. M. B. is constant: 1/3.8, regardless of the effect-duration. In agreement with these values

Table 3.

Relation between the doses of O. M. B. and O. D. P. required to obtain vaginal cornification of certain durations in mice and rats.

Effect. days	μg benzoate mice	μg diprop. mice	O. D. P. O. M. B.	μg benzoate rats	μg diprop. rats	O. D. P. O. M. B.
7.5	0.93	0.25	1/3.7	100	3.5	1/29
15.0	13.80	3.60	1/3.8	2000	27.5	1/71
30.0	218.00	57.50	1/3.8	(40000)	229.0	(1/174)
60.0	3312.00	912.00	1/3.7	(630000)	1820.0	(1/316)

the curves in fig. 2 (mice) are parallel, whereas those in fig. 1 (rats) are not.

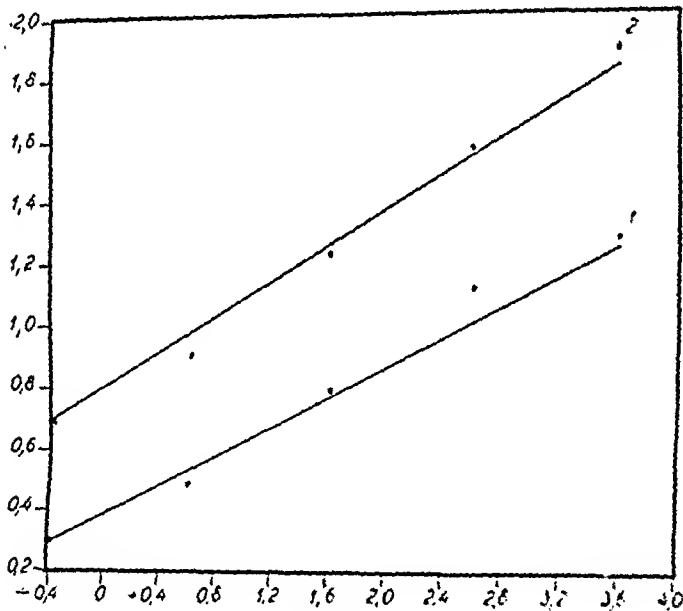


Fig. 1.

Relation between the logarithm of the dose in μg and that of the number of days in which spayed rats have positive vaginal smears after one injection of oestradioldipropionate (2) and oestradiolmono-benzoate (1). Abscissa: Logarithm of the dose in μg . Ordinate: Logarithm of the number of days with positive vaginal smears.

On the other hand, if we require an expression of the duration of the same quantities of O. M. B. and O. D. P., we see that for both mice and rats there is a constant relation, for mice approximating 1/1.5, which means that the effect of

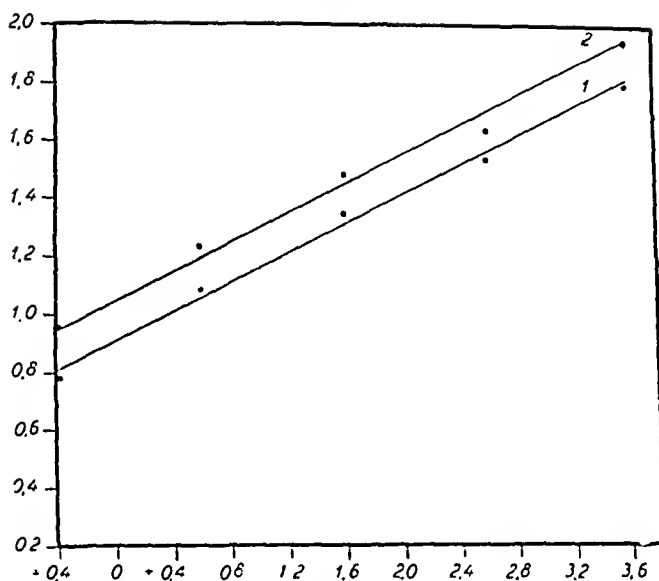


Fig. 2.

Relation between the logarithm of the dose in μg and that of the number of days in which spayed mice have positive vaginal smears after one oil injection of oestradioldipropionate (2) and oestradiolmonobenzoate (1). Abscissa: Logarithm of the dose in μg . Ordinate: Logarithm of the number of days with positive vaginal smears.

O. D. P. lasts about 1.5 times longer than O. M. B. For rats the relation is about $1/3$, meaning that the effect of O. D. P. lasts about three times as long as O. M. B. This agrees with the fact that the curves in figs. 1 and 2 are all straight lines, parallel for mice but not for rats.

3) Duration of effect measured by muscle activity test.

Our experiments on measuring muscle activity were performed on spayed female rats by means of Bugbee & Simmond's method (1926) as modified by Hemmingsen (1933). The rat is placed in a cage connected with a rotating wheel, to which the rat has unhindered access. A cyclometer is placed on the wheel to register the number of revolutions in both directions. The circumference of the wheel is 1 metre, so that one revolution corresponds to that distance. Before experi-

menting on the animal a determination is made of the number of metres travelled by it in the course of 24 hours. For young adult spayed rats the distance was a few hundred metres a day.

After being injected with large quantities of oestrogenic substance the rat will increase the distance travelled by about ten times, i. e. instead of running a few hundred metres it now runs some kilometres in a day. We then determined the number of days in which the rat displayed increased muscle activity and also the number of revolutions it runs in the period of increased muscle activity, as well as the average number of revolutions in the period. The results are summarized in Tables 4 and 5.

Table 4.

Muscle activity of adult spayed female rats after receiving the substance in one oil injection.

Dose µg	No. of days	Oestradiolbenzoate		No. of days	Oestradioldipropionate		
		Total revs.	Total revs. per day		Total revs.	Total revs. per day	Monob. Diprop.
1	4	5861	1465	11	19309	1755	1/2.5
10	9	8392	932	20	19353	2468	1/2.3
100	18	18872	1048	31	72843	1777	1/2.3
1000	30	31498	1050	72½	231486	3171	1/2.3
10000	73	92083	1261	173	432577	2500	1/2.3

From Table 4 it will be seen that the number of days in which the rat displays increased activity is practically doubled every time the dose is multiplied by 10. The same increase is found if we add together the total number of metres travelled. On the other hand, the daily distance travelled is fairly constant as far as O. M. B. is concerned and is about 1 km. For O. D. P. the level seems to lie rather higher, about 2½ km. per day. The results are shown graphically in fig. 3.

By means of the parallel curves in fig. 3 we calculate the relation between the strengths of O. M. B. and O. D. P., measured in terms of muscle activity at 1/2.3, i. e. that the same

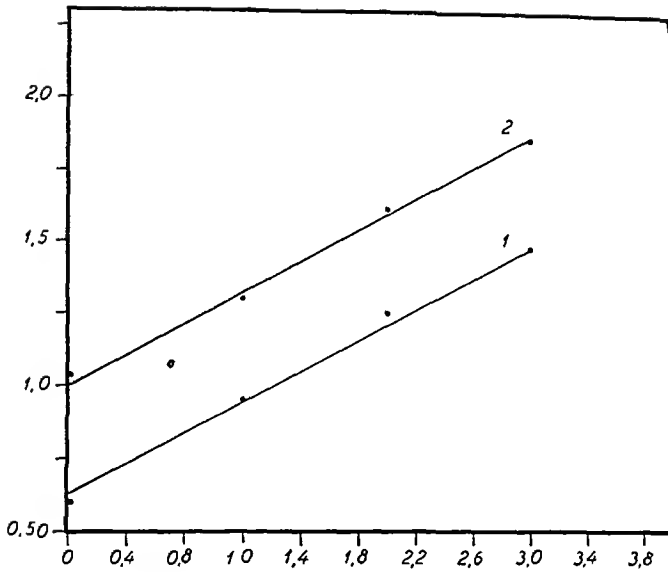


Fig. 3.

Relation between the logarithm of the dose in μg and the logarithm of the number of days in which rats display increased muscle activity after receiving oestradiolmonobenzoate (1) and oestradioldipropionate (2) in one oil injection. Abscissa: Logarithm of the dose in μg . Ordinate: Logarithm of the number of days with increased muscle activity.

weight-volume of O. D. P. produces a muscle activity lasting 2.3 times as long as O. M. B. does. If with the aid of fig. 3 we find the doses of O. M. B. and O. D. P. which produce the same effect-duration of the muscle activity, we arrive at the values shown in Table 5.

Table 5.

Relation between the doses of oestradiolmonobenzoate and oestradioldipropionate required to produce a certain duration of muscle activity (read from the curves in fig. 3).

Effect days	μg monobenzoate	μg dipropionate	$\frac{\text{dipropionate}}{\text{monobenzoate}}$
7.5	7.59	0.31	1/24.4
15.0	83.2	3.71	1/22.5
30.0	871.0	43.7	1/20.0
60.0	9120.0	501.2	1/18.0

From Table 5 it will be seen that the relation between the doses of O. D. P. and of O. M. B. required to produce the same effect duration as expressed by muscle activity in contrast to the effect duration expressed by vaginal cornification is fairly constant at 1/20.

4) *Resorption of oil and oestrogenic substance from subcutaneous depots.*

We injected subcutaneously 1/2 ml. sesame oil containing the oestrogenic substance into 10 rats and 20 mice per dose. Ten days after the injection the animals were killed and the residual oil depot was withdrawn with a syringe and filtered through dry filter paper. The syringe and filter were then washed with ether. The ether was removed from the aggregate filtrate by heating, whereupon the volume of the oil was measured. The content of oestrogenic substance was titrated on mice and rats, ten animals per dose being employed. The results are shown in Table 6.

Table 6.

Resorption of oil and oestrogenic substance in spayed rats and mice ten days after one subcutaneous injection of 0.5 ml.

Animal	Substance	Dose µg	residual oil		residual oestrogenic substance	
			ml	per cent	µg	per cent
Rats	O. D. P.	40	0.11	22	1.6	1.0
"	"	400	0.13	26	21.6	5.4
"	"	4000	0.095	19	153.5	3.9
Rats	O. M. B.	40	0.12	24	0.2	0.5
"	"	400	0.12	24	0.8	0.1
"	"	4000	0.11	22	11.0	0.3
Mice	O. D. P.	4	0.29	58	0.57	14
"	"	40	0.26	52	4.10	10
"	"	400	0.24	48	85.00	21
Mice	O. M. B.	4	0.34	68	0.13	3.3
"	"	40	0.31	62	1.50	3.8
"	"	400	0.23	46	14.00	3.5

The quantity of oil recoverable after ten days is about 2.4 times larger for mice than for rats. It will also be observed that the residual oestrogenic substance in the oil depot is fairly constant for both animals when the calculation is made as a percentage of the injected quantity and is quite independent of the size of the doses. Therefore it is permissible to make an over all comparison of the resorption from the subcutaneous oil depots containing different weight-volumes of the same substance. In the experiments referred to in Table 6 the doses is given to rats were ten times as large as those given to mice, because the quantities injected must be large enough to provide sufficient substance for titration ten days later. Although the mouse is two to four times more sensitive to these oestrogens than the rat, Tables 2 and 3 show that, at any rate with O. D. P., rats must be given doses about ten times as large as those given to mice in order to obtain the same effect-duration. As far as the rat is concerned, after the ten days there is about 14 times as much O. D. P. as O. M. B. left in the subcutaneous oil depot. With the mouse, four times as much O. D. P. is found to be unresorbed as O. M. B., which means to say that O. M. B. is resorbed much more readily from the subcutaneous oil depot than O. D. P. As regards O. D. P., the relative unresorbed quantity of substance averages three times more in mice than in rats, whereas for O. M. B. the average is ten times more.

DISCUSSION

It is quite evident from the results recorded above that O. D. P. in relatively large doses possesses a much greater protracted oestrogenic effect than O. M. B., whereas in doses just capable of inducing oestral reaction in spayed rats and mice it is less active than O. M. B. On the other hand, it is impossible to say how much more active O. D. P. is than O. M. B., such a comparison being dependent on the dose used, the test employed for arriving at such a decision, and whether rats or mice are employed. The question is also complicated by the

fact that the curves of O. M. B. and O. D. P. are not parallel when rats are employed in the tests, but only when the animals are mice.

If it is desired to know how much more O. M. B. than O. D. P. must be used to keep the animals in the oestral state for a given period, it is found that for the whole of the dosage interval employed we must use four times as much O. M. B. as O. D. P. when mice are the animals, whereas in corresponding dosage intervals for rats we require 29 to 346 times more O. M. B. than O. D. P. If muscle activity is employed as the basis of determination, we find that 20 times as much O. M. B. as O. D. P. must be used.

If on the other hand we require to know for how many days the animals can show positive vaginal smears when dosed with the same quantities of O. M. B. and O. D. P., we find that O. D. P. is 1.5 times as active as O. M. B. in mice and three times as active in rats. Employing muscle activity as the criterion, we find that O. D. P. is 2.5 times as active as O. M. B.

Thus when we inject large doses of O. D. P. and O. M. B. into mice and rats we get all possible proportions from 1.5 to 346. What the position is in the female human organism can therefore only be determined by experiments upon ovariectomized or post-climacterial women. However, there can hardly be any doubt but that here again large doses of O. D. P. will prove to be superior to O. M. B.

The tests of the concentration of oestrogenic substance in the oil depots after a certain period showed that the concentration of the residual oestrogenic substance expressed as a percentage of the quantity injected is fairly constant for both mice and rats. This might mean that in the living organism the oestrogenic substance passes from the oil phase to the water phase according to the same laws as those governing between two mutually immiscible phases.

SUMMARY

Applied in *small doses*, oestradiol monobenzoate (O. M. B.) is more active than oestradiol dipropionate (O. D. P.) in both spayed mice and spayed rats when the substances are injected in oil solution, and the effect is measured by means of the vaginal cornification test.

On the other hand, measured by the same test O. D. P. in *larger doses* produces a much more protracted effect in both mice and rats than O. M. B. The same effect-duration is obtained in mice with O. D. P. and O. M. B. in the proportion of $\frac{1}{4}$ throughout the dosage interval tested. For rats, on the other hand, the longer the duration is desired, the more O. M. B. must be used than O. D. P. For an effect of $7\frac{1}{2}$ days, 29 times more O. M. B. than O. D. P. must be injected, and for a 15 days' effect 71 times more.

Measured by muscle activity on rats, the same effect duration is obtained with 1 part of O. D. P. as compared with 20 parts of O. M. B., regardless of the whole of the dosage interval.

Applied in *larger doses* the same doses of O. D. P. in mice produce an effect 1.5 times as long, in rats three times as long as O. M. B., measured by the vaginal cornification test. Measured by muscle activity on rats the same doses of O. D. P. produce an effect lasting about 2.5 times as long as O. M. B.

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COLORIMETRIC ASSAY OF TESTOSTERONE

BY

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This paper extends previously published work (*Theil Nielsen*, 1948) concerned with the quantitative assay of androgens in simple solutions. It describes a convenient procedure for the determination of testosterone. The work was undertaken with the expectation that such data will be of value in the development of assay methods applicable to more complex solutions, e. g. pharmaceutical preparations, extracts of biological materials etc.

Since the discovery of testosterone, very few suitable chemical methods have been published for the quantitative determination of this hormone. Heretofore, the colour reaction proposed by *Koenig et al.* (1941) has been the only relatively specific colorimetric method for testosterone.

While studying the sulphuric acid method for dehydroandrosterone (*Theil Nielsen*, 1948) the writer observed that, under certain conditions, testosterone and the closely related androstenedione (3,17) gave a strong colour reaction with the sulphuric acid reagent. Below is an attempt at adapting the reaction to quantitative purposes.

EXPERIMENTAL

1. *Principle of method.*

Two modifications will have to be described:

a. A test-tube (180×20 mm.) containing the testosterone (dry) is placed in an ice bath. 1 ml. sulphuric acid is added and mixed with a stirring rod. The tube is heated in boiling water for 5 minutes, and then replaced in an ice bath. Next 4 ml. of 25 per cent. alcoholic sulphuric acid (one volume sulphuric acid diluted with three volumes ethanol) are added. After thorough stirring the mixture is placed in a thermostat at 29° for 30 minutes. After being transferred to a photometer cell (1 cm.) the extinction is read at 600 m μ . The blank consists of reagents without testosterone.

b. In this modification the testosterone is dissolved in 0.2 ml. ethanol prior to the addition of sulphuric acid. The mixture is heated in boiling water for 30 seconds. For the rest, the test is carried out as described under *a*.

2. Absorption spectrum of the reaction colour.

Fig. 1 is a graphic representation of an experiment with 12.5 and 25 μ g. testosterone. Owing to the strong absorption in the 600 m μ range the reaction colour is pure blue. Actually, to the naked eye, it is quite indistinguishable from that given by dehydroandrosterone in the above mentioned colour test for that substance. This fact, of extreme importance when analyzing mixtures containing both substances, will be discussed below.

3. Heating time.

A series of experiments was carried out according to the principle given in section 1, *b*, but with the difference that the heating time was varied from 10 seconds to 5 minutes. The amount of testosterone was 10 μ g. The result will be seen in fig. 2, showing that the colour intensity reaches its maximum value after approximately 30 seconds of heating.

Similar experiments carried out according to 1 *a* (dry testosterone) gave maximum colour after 3—5 minutes heating. The heating time in relation to the specificity of reaction will be gone into later.

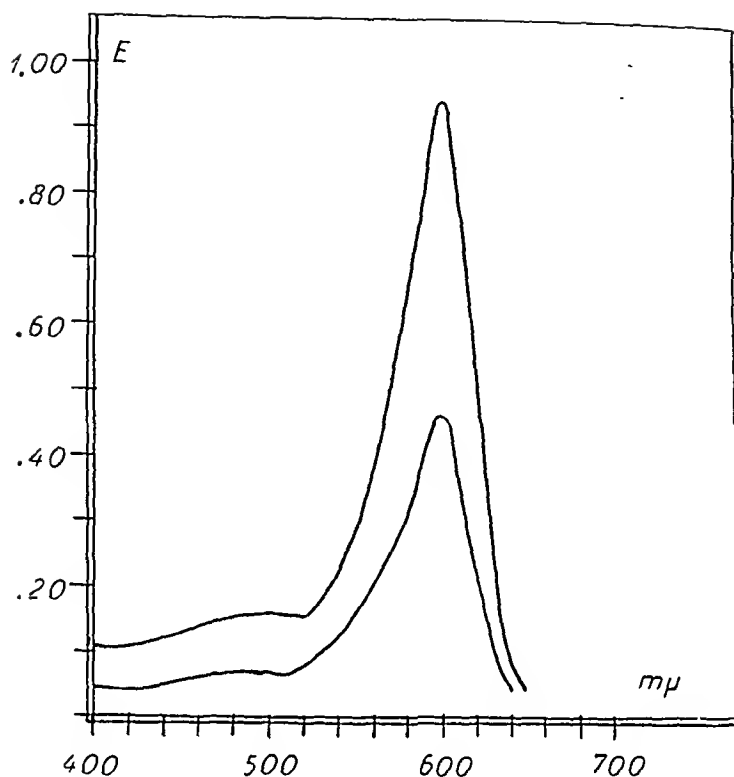


Fig. 1.

Absorption spectra for testosterone (12.5 and 25 $\mu\text{g.}$) after sulphuric acid reaction.

Abcissa: Wave length in $\text{m}\mu$.

Ordinate: Extinction coefficient.

4. *Dilution medium.*

In this connection two matters of importance must be studied:

- α) Kind of solvent (water or ethanol),
- β) Acid concentration in the solvent.

While elaborating the present method it soon appeared to us that ethanol had an important bearing upon the reaction. This will be seen from the following experiments.

Five test-tubes, each containing 25 $\mu\text{g.}$ testosterone, were heated for 5 minutes (boiling water) with 1 ml. sulphuric acid. After cooling and diluting with 4 ml. 25 v/v per cent. sul-

phuric acid (aqueous), different amounts of ethanol (0.1 to 0.8 ml.) were added. After standing at 40° for one hour the extinction at 600 m μ was read. The experimental results will appear from fig. 3. These results stress the importance of the content of ethanol in the dilution medium and naturally

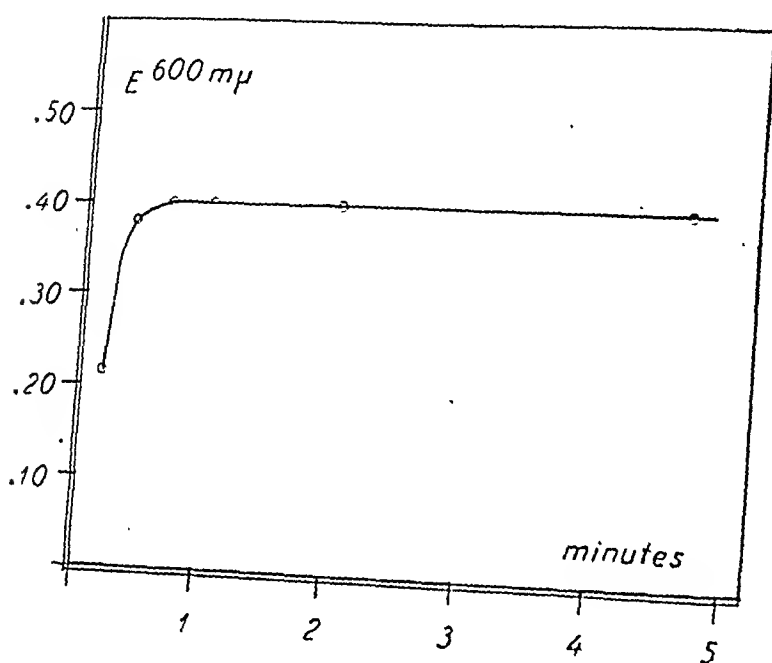


Fig. 2.

Effect of heating (boiling water) upon colour intensity.

Abscissa: Heating time in minutes.

Ordinate: Extinction coefficient, at 600 m μ .

brought a similar experiment with pure alcoholic sulphuric acid (1 volume sulphuric acid mixed with 3 volumes ethanol). In this case the extinction coefficient was found to be as high as 0.81. It was decided, therefore, in the following to use the last-mentioned sulphuric acid reagent. The next problem to be solved is that of the influence of the acid concentration upon the colour development. Serving this purpose the following experiments were set up: Identical amounts of testosterone (25 μ g.) were heated for 5 minutes (boiling water) with 1 ml. sulphuric acid. After cooling in an ice bath dilution was undertaken with 4 ml. alcoholic sulphuric acid of different strengths

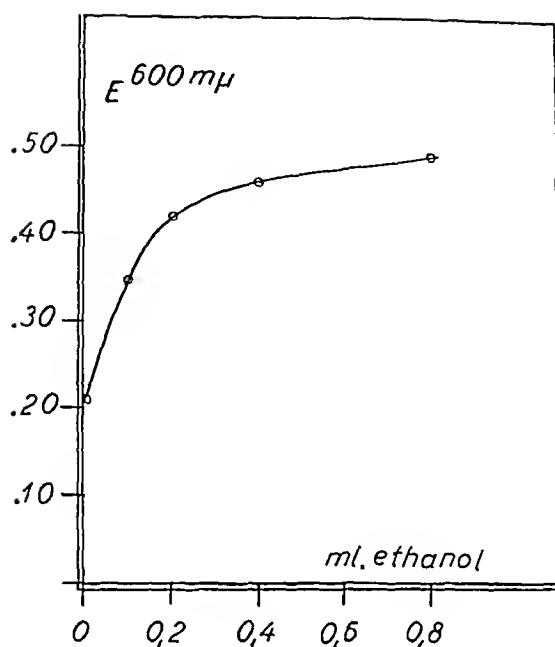


Fig. 3.

Effect of ethanol upon colour development.
 Abscissa: ml. ethanol added to dilution medium.
 Ordinate: Extinction coefficient at 600 m μ .

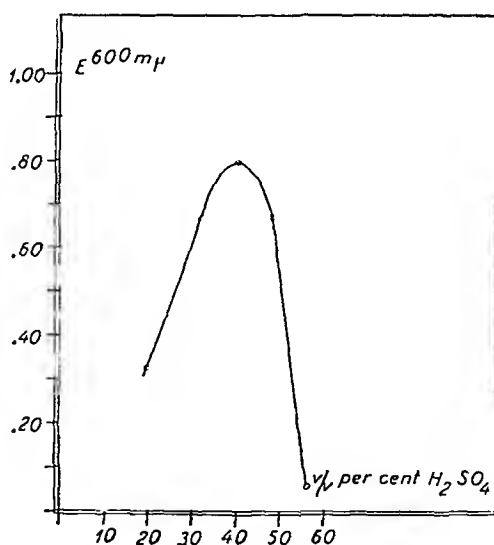


Fig. 4.

Influence of acid concentration upon colour formation.
 Abscissa: Content of sulphuric acid (volume/volume per cent)
 in the reaction mixture.
 Ordinate: Extinction coefficient at 600 m μ .

in order to obtain the following concentrations of sulphuric acid, expressed in v/v per cent:

- 1) 20 per cent.
- 2) 32 » »
- 3) 40 » »
- 4) 48 » »
- 5) 56 » »

The mixtures were kept at 40° for 30 minutes, whereupon the extinction at 600 mμ was read. In fig. 4 the colour intensities are plotted against the acid concentration. It will be seen that a content of approximately 40 per cent. sulphuric acid gives maximum colour formation. This means that dilution will have to proceed with 4 ml. of a mixture of one volume sulphuric acid and three volumes ethanol.

5. *Rate of colour development:*

This question has been studied only to a small extent. At room temperature, 22—30°, maximum colour is obtained within 15—25 minutes after admixture of the alcoholic sulphuric acid reagent. Fig. 5 demonstrates this fact. The experiment was carried out at 29°. Colour intensity is plotted against the time (in minutes) elapsing after the addition of alcoholic sulphuric acid.

6. *Stability of reaction colour.*

The colour is stable for at least one hour.

7. *Relation between extinction coefficient and amount of testosterone.*

This question must be subjected to more detailed comment. Firstly, the reader's attention is drawn to section 1. It will be seen that the reaction can be carried out in two different ways: The sulphuric acid may be added to the testosterone, this latter being either dry or dissolved in 0.2 ml. ethanol. Experiments showed that it is of some importance whether ethanol is used or not. The colour intensity obtained with testosterone dissolved in 0.1—0.2 ml. ethanol is definitely

higher than that given by the dry substance, so that different calibration curves result according as testosterone is dissolved in ethanol or not. This will appear from fig. 6, showing calibra-

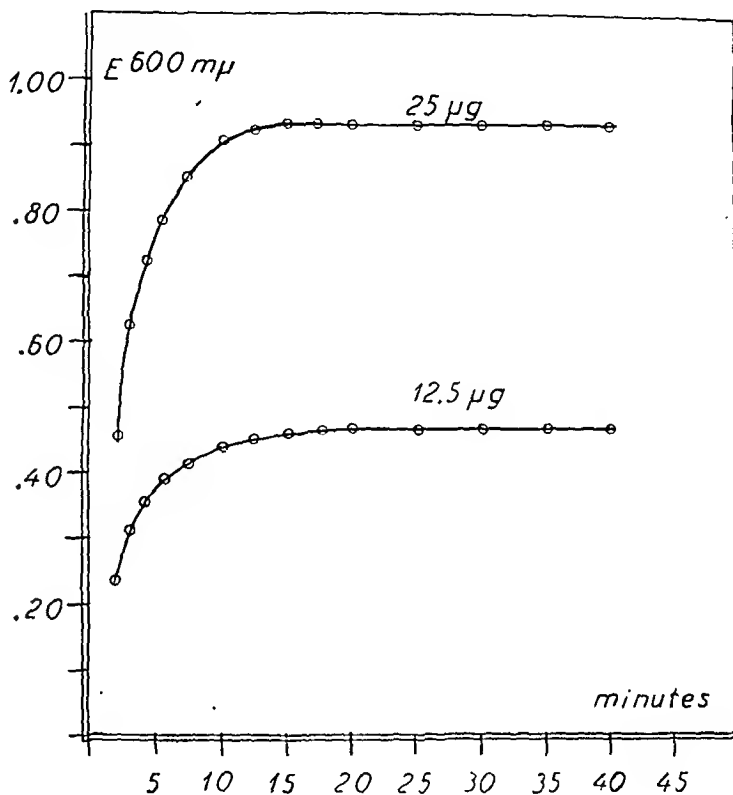


Fig. 5.

Rate of colour development at 29° .

Abcissa: Time in minutes after admixture of alcoholic sulphuric acid.

Ordinate: Extinction coefficient at $600\text{ m}\mu$.

tion curves for testosterone subjected to the sulphuric acid tests as specified in section 1 *a* and *b*. Using larger amounts of ethanol we obtain even higher extinction values; but, possibly because of uncontrollable evaporation of ethanol during the initial heating, the results are too variable and irreproducible. Looking at the curves in detail we notice that the colour reaction does not comply entirely with Beer's law. Fortunately, it has appeared that from day to day the readings

are subject only to small variations. Therefore, it will suffice to check the curves at adequate intervals. The question may be asked why two different methods are proposed. The answer is

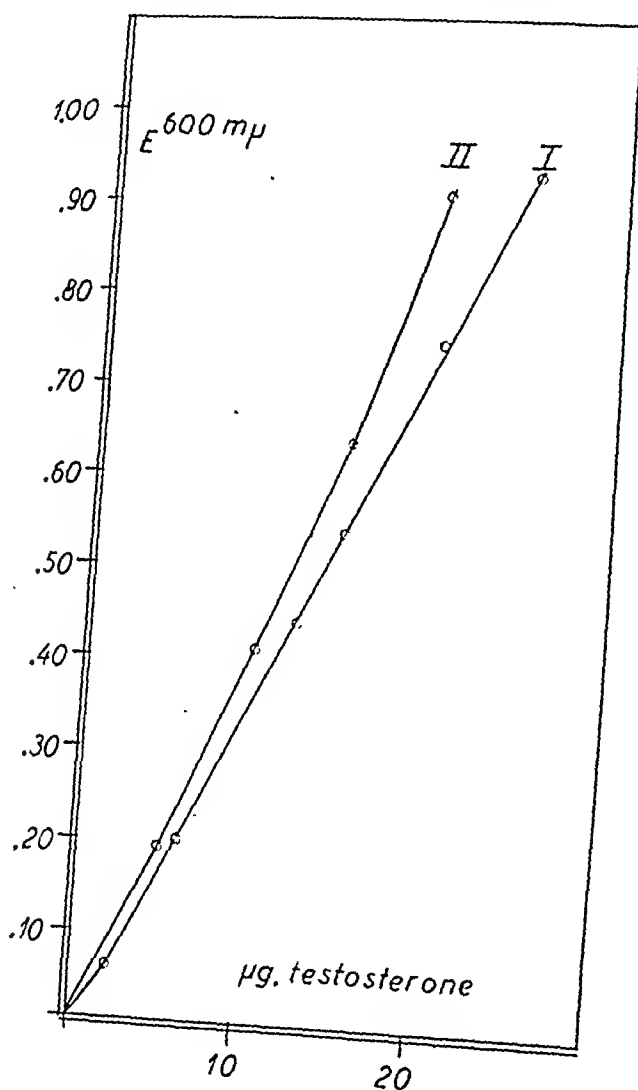


Fig. 6.

Calibration curves for testosterone.

Curve I: Dry testosterone.

Curve II: Testosterone dissolved in 0.2 ml. ethanol.

Abcissa: Amount of testosterone in μg.

Ordinate: Extinction coefficient at 600 mμ.

that using testosterone in the dry state (modification a) a more accurate and reproducible reaction is obtained than when

using an alcoholic solution of the substance. This may, perhaps, be ascribed to the aforementioned evaporation of ethanol. In certain practical applications, however, the ethanol (modification *b*) proves useful and convenient (see below).

8. *Accuracy of method.*

Our material is not yet large enough to justify real statistical treatment. For that reason we must confine ourselves to state that the experimental errors seem to be of the same order of size as in most similar methods of determination, i. e. about ± 5 per cent, depending on the errors that are associated with pipetting the small volumes of fluid.

9. *Specificity of reaction.*

Undoubtedly, this question is one of the most important to be dealt with. From the very start, however, it must be emphasized that the vast number of steroid hormones known at the present time excludes the exhaustive examination of this problem.

The following substances are found to give insignificant or no reaction:

Androsterone,	Cholesterol,
Isoandrosterone,	7-Dehydrocholesterol,
Δ^5 -Androstene- 3β , 17-diol,	7-Hydroxycholesterol,
Methyltestosterone,	7-Ketocholesterol,
Progesterone,	Cholestenone.
Oestrone,	
Oestradiol,	
Equilin,	
Equilenin.	

At least 3 substances give a colour reaction very similar to that given by testosterone. In the following we shall give an account of the reactions which, under the conditions described in section 1, are obtained with these three substances:

a. Δ^4 -Androstene- 3β , 17-dione.

When subjected to the sulphuric acid reaction, andro-

stenedione produces a reaction colour entirely identical with that given by testosterone, in respect of intensity, and spectral composition. Yet, this substance can be effectively removed by means of nicotinic acid hydrazide. (Vélluz & Pétit, 1945).

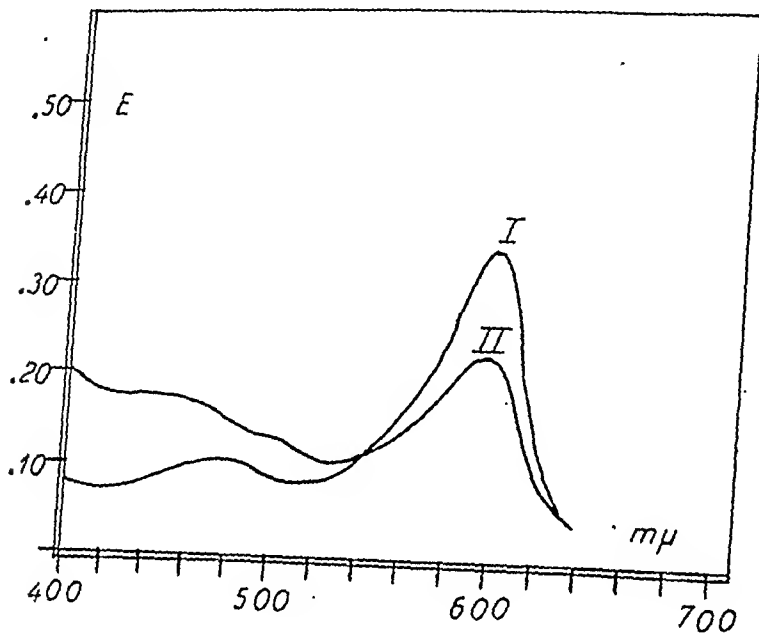


Fig. 7.

Desoxycorticosterone. Showing the effect of prolonged heating upon colour formation. 25 μ g. were subjected to colour reaction according to the directions given in section 1, b, but with different times of heating.

Curve I: 1 minutes heating.

Curve II: 1 hour's heating.

Abscissa: Wave length in $m\mu$.

Ordinate: Extinction coefficient.

b. *Desoxycorticosterone*.

This hormone, too, gives a blue colour when treated with the sulphuric acid reagents. The extinction at 600 $m\mu$, however, is considerably lower than that given by equal amounts of testosterone. In addition, it may be further decreased by extending the heating time from one minute (modification 1, b) to one hour. This will appear from fig. 7.

c. *Dehydroandrosterone*.

According to our earlier investigations (Theil Nielsen, 1948)

it is to be expected that this substance will react positively. Still, as in the case of desoxycorticosterone, extension of heating time decreases the colour intensity obtained by dehydroandrosterone. This fact is clearly demonstrated by the following

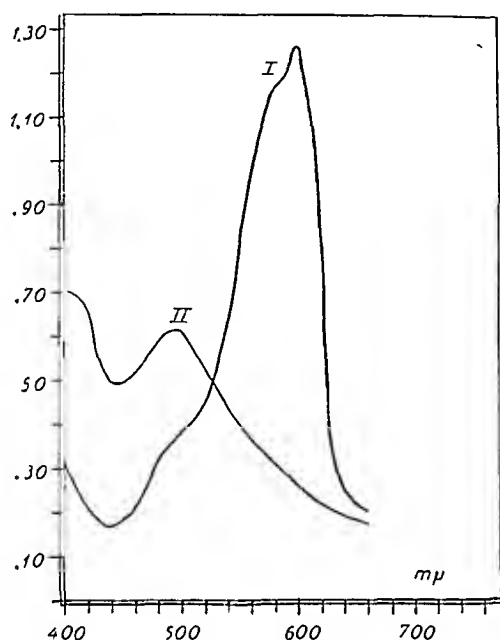


Fig. 8.

Dehydroandrosterone. Effect of prolonged heating upon colour intensity. 100 μ g. were treated as described in section 1 b, but with different times of heating.

Curve I: 1 minutes heating.

Curve II: 1 hour's heating.

Abscissa: Wave length in mμ.

Ordinate: Extinction coefficient.

experiment: 100 μ g. dehydroandrosterone were treated according to modification 1 b. A second sample was treated in the same way, but with the difference that the heating time was extended to one hour. The absorption spectra from this experiment will be seen in fig. 8. It appears that one hour's heating almost entirely prevents the specific colour formation. Similar experiments carried out with testosterone proved that in this case the colour was unaffected by heating for one hour.

Accordingly, it seems very easy to prevent the influence of dehydroandrosterone upon the colour reaction.

10. *Practical applications of the method.*

This problem has so far been studied only as regards simple pharmaceutical preparations of testosterone or testosterone propionate. It is, however, necessary to point out that in this field our research is by no means complete. Therefore, we are unable to give more than the simple outlines of our work.

The most common preparations for clinical use are oily solutions of testosterone propionate. Such preparations have been analyzed in the following manner: 1 ml. of the oily solution is heated with 5 ml. 2 N alcoholic potassium hydroxide till complete saponification. Subsequently 50 ml. water are added. The solution is extracted twice, each time with 75 ml. ether. After washing with 50 ml. N sodium hydroxide and 50 ml. water the combined ether extracts are evaporated to dryness. The solids are dissolved in a sufficient amount of ethanol and subjected to analysis as described in section 1, *b*. Blank experiments carried out with different kinds of oil (without testosterone) showed a chromogenic value corresponding to about 0.5—1 mg. testosterone propionate per ml.. In the case of solutions containing large amounts of this substance (e. g. 25 mg. per ml.) the colour originating in the oil itself is without real significance. Accordingly, our experiments have shown 100 per cent. recovery of testosterone propionate when oily solutions of this strength have been analyzed. When we are dealing with weaker solutions (5—10 mg. per ml.) the influence of the oil upon colour formation is correspondingly stronger, and in such cases it will perhaps be necessary to use more complicated purification procedures. Finally, it should be noted that the modification of analysis given in section 1, *b* is the only one applicable to the determining of testosterone isolated from oily solutions in the aforementioned manner. When applying modification 1, *a* the blank values are considerably higher because of intense browning following the addition of sulphuric acid directly to the dry

saponification residue (instead of an alcoholic solution of the latter).

SUMMARY

A simple and sensitive method is proposed for the quantitative determination of testosterone.

The procedure, described in detail in section 1, is based upon the blue colour formed when testosterone is heated with sulphuric acid and subsequently mixed with 25 v/v per cent. alcoholic sulphuric acid.

Certain aspects related to specificity are discussed.

The practical application to the analysis of pharmaceutical preparations is roughly outlined.

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»MICRO-METHODS« FOR THE DETERMINATION OF 17-KETOSTEROIDS IN URINE

BY

CHRISTIAN HAMBURGER

WITH A STATISTICAL APPENDIX BY G. RASCH

The common methods for the determination of 17-ketosteroids in urine require the extraction of a urine volume equal to a half or a quarter of a 24-hour specimen. The colour reaction, however, is actually made with an amount of extract from 1/100 to 1/200 of the 24-hour urine; the rest is discarded unless a separation of the various 17-ketosteroids is desired. In most clinical assays the determination of the total neutral fraction is sufficient and it is, therefore, tempting to save chemicals and space by extracting merely an amount of urine necessary for the colour reaction. *Drekter et al.* (1947) have shown that the analysis can be performed satisfactorily with only 10 ml. of urine.

The time required for the extraction can also be reduced considerably even without the use of more or less complicated extractors. *Robbie & Gibson* (1943) thus found that at least 90 per cent of the 17-ketosteroid content are removed from the previously hydrolyzed urine (250 ml.) by 10-minute carbon tetrachloride extraction in a boiling waterbath.

Before the adoption of the »micro-methods« to be described in the present communication, our standard routine method consisted in simultaneous sulphuric acid hydrolysis and ben-

zene extraction involving three 1½-hour refluxings (for details, see *Hamburger*, 1948 a, b). The long time required for this procedure and the limited number of steam-boilers at our disposal forced us to try simpler methods. We then had to make our choice between several possibilities as to the amount of urine, the kind of acid and solvents, etc.

CHOICE OF REAGENTS, EXTRACTION PROCEDURES AND AMOUNTS OF SOLVENTS

1) *Amount of urine.*

We have adopted Callow's modification of the Zimmermann-reaction and perform a duplicate test with an amount of alcoholic extract equal to 1/200 of a 24-hour urine. This amount enables us to measure a content of up to 25 mg./24 hrs. With the intention of being able to determine higher 17-ketosteroid contents without making a new extraction we have preferred to work with 1/50 of a 24-hour urine. If this amount happens to be less than 10 ml., water is added ad 10 ml., because it is inconvenient to handle smaller volumes.

2) *Acid for the hydrolysis.*

Most investigators have used hydrochloric acid. The shortage of glass ware during the war and until recently necessitated the use of platinated metal reflux condensers which did not stand the HCl-vapours. Consequently, the urines were acidified with sulphuric acid, and we have not been able to demonstrate any difference between the effect of these acids upon the hydrolysis, provided such amounts are chosen as bring forth the same pH-value. In the standard routine method concentrated H_2SO_4 was used; when dealing with the »micro-tests« a corresponding larger amount of 40 volume per cent H_2SO_4 is more convenient.

3) *Optimal pH-value.*

The optimal pH-value, i. e. the pH which gives the largest

recovery of total 17-ketosteroids, varies with the time of hydrolysis and with the temperature.

We commenced a thorough investigation but soon found that the optimal value varied from one batch of urine to another. All pH-measurements were made with a glass electrode. In some specimens the largest recoveries occurred at pH 0.40–0.60 (15 minutes' boiling on electric hot plate), in other urines in the neighbourhood of pH 0. On the average the optimal pH-value was about 0.20 or slightly below this figure. This fact clearly demonstrates that it is not possible to make general statements of the optimal pH-value, and since for practical reasons it is not feasible in the clinical routine analyses to perform the analysis at a variety of pH-values, we decided to use a constant acid concentration for all urines, viz. 10 volume per cent of 40 per cent H_2SO_4 . This concentration brings on a average the pH to about 0.20.

4) Time of hydrolysis.

Callow et al. (1939) examined the effect of time of boiling urines with 40 ml. of HCl per litre on the liberation of 17-ketosteroids. There were some irregularities, but the type of curve given by men's and women's urine seemed to differ significantly. We have made some experiments on the effect of varying the time of boiling when the acid concentration and the temperature were kept constant. Three of these experiments are summarized below; in all instances 1/50 of a 24-hour urine was used, and after acidification with 10 volume per cent of 40 per cent H_2SO_4 the urines were submitted to gentle boiling on electric hot plate.

- 1) Man, 44 years old. The urine (pH 0.20 after acidification) was boiled for 5 to 40 minutes. After chilling it was extracted with ether in a separatory funnel. As evident from Fig. 1 a, the largest recovery occurred after 25 minutes.
- 2) Woman, 57 years old. The urine (pH 0.30 after acidification) was the first 24-hour specimen after intramuscular injection of 200 mg. of testosterone propionate in oily solution; it was boiled for 10 to 40 minutes and afterwards extracted with ether in a separatory

funnel. The maximal recovery was found at 40 minutes, but the difference between this and the 25-minute value does probably not exceed the experimental error (see Fig. 1 b).

- 3) Woman, 46 years old. The pH of the acidified urine was 0.20. The urine was hydrolyzed for 5 to 40 minutes and immediately afterwards extracted with 50 ml. of carbon tetrachloride on the hot plate for 30 minutes. The values shown in Fig. 1 c represent the average figures for 3 tests. The maximal recovery was obtained after 20 minutes' hydrolysis, but it is reasonable to assume that the hydrolysis has continued for some time during the subsequent refluxing with CCl_4 until the liberated 17-ketosteroids have passed to the extraction solvent, probably in about 5 minutes.

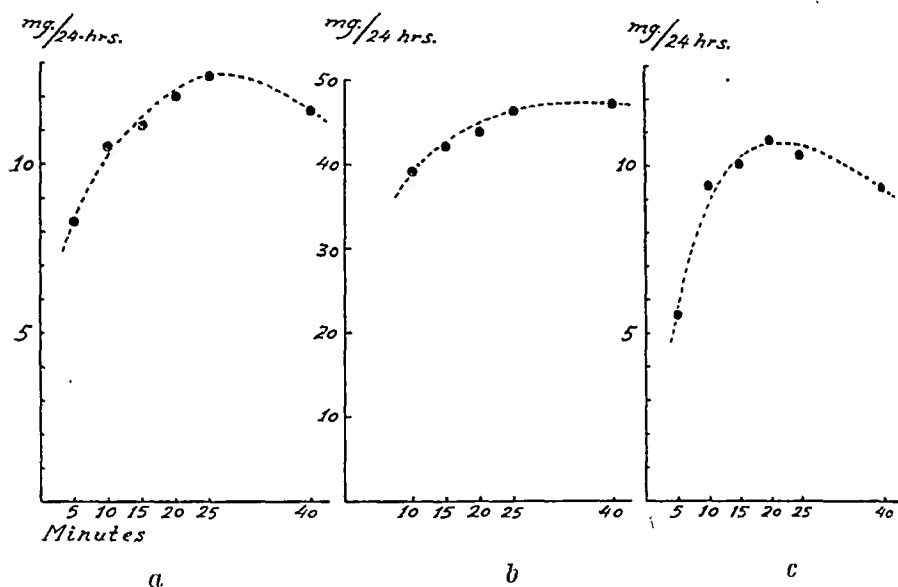


Fig. 1.

The effect of time of acid hydrolysis upon the recovery of 17-ketosteroids from three batches of urine.

These, and other experiments seem to indicate that under the present experimental conditions, the optimal time of boiling is about 25 minutes.

5) Solvent for extraction.

It is outside the scope of this paper to evaluate the numerous communications dealing with the choice of solvent for the extraction. We shall confine ourselves to emphasize that when the opinions of the superiority of benzene, ether or

carbon tetrachloride differ so much, this fact might indicate that the difference between these solvents with regard to their ability to extract 17-ketosteroids is actually rather small. In our hands CCl_4 has on an average given somewhat lower recoveries than benzene or ether and the technical variation seems to be somewhat larger when using CCl_4 . Furthermore, the high specific gravity of CCl_4 constitutes a practical drawback for the purification of the extract. Because it forms the bottom layer, several separatory funnels are needed for each urine, or the washings must be done in flasks of special design and the supernatant layer removed by aspiration (Wooster, 1943). In spite of the unquestionable advantage of working with a non-inflammable reagent we have preferred to use benzene or ether.

6) *Separate versus combined hydrolysis and extraction.*

When dealing with large volumes of urine and long-continued extraction, Callow *et al.* (1939) found that separate HCl-hydrolysis followed by benzene extraction in a continuous extraction apparatus, and the combined method of Dingemans, Borchardt & Laqueur (1937) were almost equally effective. Talbot *et al.* (1940) preferred the combined CCl_4 -extraction, but Robbie & Gibson (1943) and Friedgood, Taylor & Wright (1943) have given strong evidence to the fact that independent hydrolysis and CCl_4 -extraction were more efficient than simultaneous hydrolysis and CCl_4 -extraction. Our experiments with small volumes of urine confirm the experience of the last-named two groups of investigators. Two specimens of urine from a 56-year-old woman with mammary carcinoma (the 2nd and 3rd day after intramuscular injection of 100 mg. of testosterone propionate in oily solution) were submitted to the separate and combined CCl_4 -procedures.

A) Second 24-hour specimen (pH 0.29 after acidification).

- 1) Ten minutes' refluxing on electric hot plate followed by 20 minutes' refluxing with 50 ml. CCl_4 . Recovery: 13.2 mg./24 hrs.
- 2) Thirty minutes' hydrolysis + extraction with 50 ml. CCl_4 performed at the same time as 1) and on the same hot plate. Recovery: 5.8 mg./24 hrs.

B) Third 24-hour specimen (pH 0.24 after acidification).

- 1) Ten minutes refluxing followed by 30 minutes' CCl_4 -extraction. Recovery: 7.4 mg./24 hrs.
- 2) Forty minutes' hydrolysis + extraction with CCl_4 performed simultaneously with 1) and on the same hot plate. Recovery: 4.4 mg./24 hrs.

The recovery by the combined method was thus 44 and 59 per cent of that by the separate process. When using benzene, we have not been able to find any difference between the two methods. The most likely explanation might be that because CCl_4 forms the bottom layer, the temperature of the urine remains around 75°C , the boiling point of CCl_4 , whereas the urine during benzene extraction forms the bottom layer and therefore keeps a temperature of about 100°C .

As a consequence of the experiments cited above we have confined our efforts of elaborating the »micro-analyses« to two methods, viz. simultaneous H_2SO_4 -hydrolysis and benzene extraction, and independent H_2SO_4 -hydrolysis and subsequent ether extraction in separatory funnel.

TECHNIQUE

An amount of urine equal to $\frac{1}{50}$ of a 24-hour output is measured out from a 10 ml. pipette graduated to 0.1 ml. into a flask (if below 10 ml., water is added ad 10 ml.). From a 1 ml. pipette graduated to 0.01 ml. 10 volume per cent of 40 per cent H_2SO_4 are added to the urine.

1) *Simultaneous hydrolysis and benzene extraction.*

Forty ml. of benzene (»crystallisable«) are added to the acidified urine. The boiling is performed on electric hot plate in 450 ml. flat-bottomed Jena glass flasks (as those used for cultivation of tubercle bacilli) supplied with reflux condensers. An electric hot plate with a diameter of 22 cm. leaves space for three of the flasks, and the reflux condensers can be connected serially (see Fig. 2). When the temperature of the hot plate is kept moderate the urine boils smoothly, especially

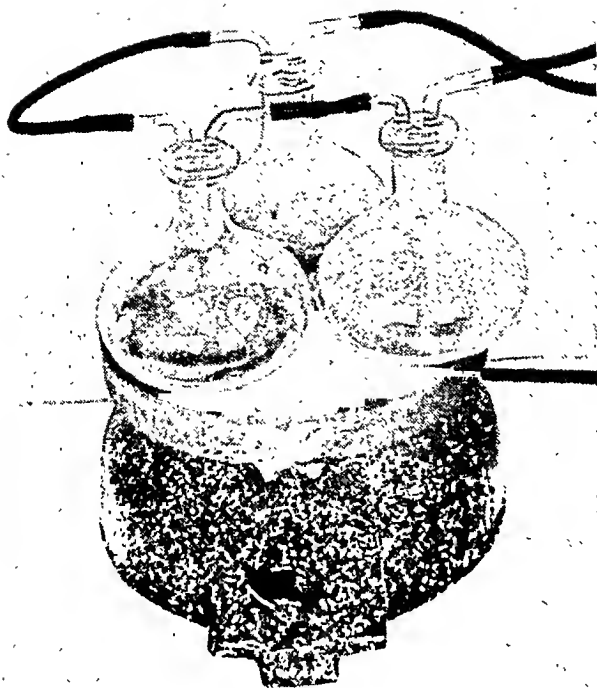


Fig. 2.

Photograph of three flasks for refluxing on an hot plate.

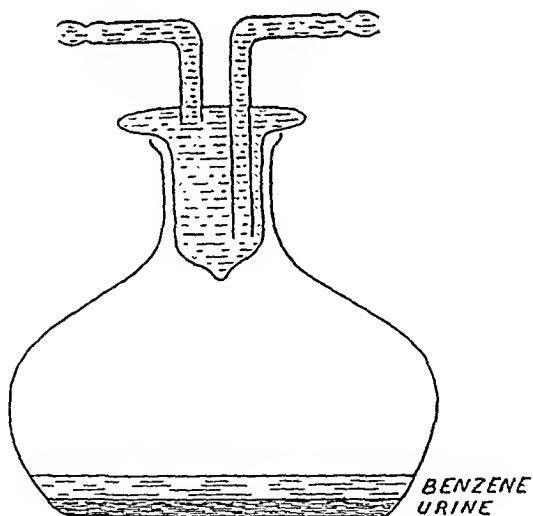


Fig. 3.

Drawing of flask and reflux condensor for benzene extraction of small urine volumes.

when a piece of glass is put into the flask. The advantage of this shape of flask is the extended contact surface between the urine and the benzene layer (Fig. 3); with 20 ml. of urine the contact surface is about 80 sq. cm. while the height of the urine layer is merely $\frac{1}{4}$ cm. The liberated steroids therefore pass very rapidly to the benzene layer. By benzene refluxing of various duration of previously hydrolyzed urine it can be shown that most of the steroids are removed within 5 minutes. Since the hydrolysis usually is complete after 25 minutes, the total time for the benzene refluxing ought to be 30 minutes. We have, in fact, found that the recovery was not increased by extending the refluxing to 40 or 60 minutes, and by re-extraction with benzene for 30 minutes the recovery is below 2 per cent of that of the first extraction. As an example mention can be made of the urine with the highest 17-ketosteroid content available for the »micro-analyses«. Re-extraction of this urine which by the first extraction gave a recovery equal to 55 mg./24 hrs. removed an additional amount of merely 1.5 per cent of this value. In our standard routine method the third benzene extract may contain as much as 9 per cent of the amount from the first and the second extract together, although each extraction took 90 minutes.

After 30 minutes' refluxing the mixture is cooled under running tap water and the benzene extract is freed from non-specific chromogenic substances and phenols by extracting once with saturated NaHCO_3 solution, twice with 2 N NaOH solution and twice with water, each of the washings being made with about 10 ml. After drying with dehydrated Na_2SO_4 , the benzene extract is filtered and evaporated to dryness over a boiling waterbath under reduced pressure. The residue is taken up in 0.80 ml. of absolute alcohol. The colour reaction is made in duplicate with 0.20 ml., an amount equal to $\frac{1}{200}$ of a 24-hour specimen (see: *Hamburger*, 1948 a, b). The rest of the alcoholic extract is saved with a view to the possibility that the content of 17-ketosteroids is above 25 mg./24 hrs. and a new colour reaction with a smaller amount has to be performed.

2) *Separate hydrolysis and ether extraction.*

Our technique is essentially identical with the method of *Drekter et al.* (1947). The hydrolysis is performed in the same flasks as used in the benzene procedure by refluxing on the hot plate for 25 minutes. The urine is cooled and extracted by vigorous shaking for one minute with 40 ml. of ether in a separatory funnel. The purification of the ethereal extract is identical with that for the benzene extract. We have found the ether method to be as effective as the benzene method; re-extraction of a urine from which the first ether extraction had removed an amount of 17-ketosteroids equal to 46.0 mg./24 hrs. gave a recovery of merely 0.8 mg./24 hrs., i. e. 1.7 per cent.

It is not easy to decide which of the two techniques, the benzene or the ether method, is the more desirable. As will be seen from the section below, the methods give the same results, and the time required for the analyses is the same. The ether method has the advantage that it is not necessary to use reduced pressure for the evaporation of the extract, and the ether removes a somewhat smaller amount of non-specific urinary chromogens, a circumstance which is, however, of minor importance, when a colour correction is used for the final calculations.

ACCURACY OF THE »MICRO-METHODS« AND COMPARISON WITH THE STANDARD ROUTINE METHOD

Special stress has been laid upon the comparison of the results obtained by the »micro-methods« and by the standard method, because our normal values (*Hamburger*, 1948 a, b) were obtained by the last-mentioned method. The accuracy of a method is quite often evaluated by particular experiments of this kind: Each specimen is divided into a certain number of equal portions and the various steps of the procedure are carried out by the same person, simultaneously and with the same

batches of reagents. The results are usually found to be very consistent. Such experiments, however, only yield an upper bound of the accuracy of the method as used in the routine. In order to determine the accuracy in routine work the control experiment must be carried out under routine conditions: In the course of some time the analyses are made in duplicate, but each of these is always performed on different days, often with different batches of reagents and not always by the same person. To state it briefly, the control tests are deliberately mixed with the bulk of the ordinary routine analyses. The investigations into the accuracy of the methods to be reported below are all made in the latter way, with one exception: the comparison of the »micro-benzene-method« and the »micro-ether-method«. In this case the purpose was to disclose any possible difference in a particular step of the whole procedure, viz. in the ability of benzene and of ether to extract the 17-ketosteroids. Therefore these analyses were carried out simultaneously and under as similar experimental conditions as possible.

So far, we have examined 61 urine specimens by the standard method and by the »micro-benzene-method«. Furthermore we have carried out 20 independent duplicate standard analyses and 50 independent duplicate »micro-benzene-analyses«. Finally, we have compared the »micro-benzene-« and the »micro-ether-method« in 28 urines. The results of the statistical analysis, the details of which are given in the appendix, may be summarized in this way:

It is not possible to demonstrate any systematic difference between the micro-methods and the standard routine method. The standard method is less accurate than the micro methods. When the amount of 17-ketosteroids is in the neighbourhood of 10 mg. per 24 hours, the standard deviation is 50 per cent lower in the micro-methods than in the standard method: accordingly, in order to obtain the same accuracy we should need 4 standard analyses for every micro-determination. Furthermore, we have found no systematic difference between the »micro-benzene-« and the »micro-ether-method«, but from

the present experiments we cannot decide which of these methods is the more accurate. The important question whether it is safe to use our »normal values« also for the micro-analyses has been answered in the affirmative sense.

STATISTICAL APPENDIX

In Fig. 4 the results of the 50 duplicate »micro-benzene-analyses« are plotted against each other. Obviously the variations about the »identity-line« increase with increasing amounts of 17-ketosteroids. In order to investigate the relative errors we plot the logarithms in Fig. 5. It is seen that for larger amounts of 17-ketosteroids the deviations from the identity line keep within fairly narrow constant limits, but for the amounts less than 1 mg./24 hrs. the variations are much larger. This indicates that two different kinds of errors are interfering, a »constant relative error« and a »constant absolute error«. For small amounts of 17-ketosteroids the constant relative errors are insignificant as compared with the »absolute errors«, but for larger amounts the contribution of the »absolute errors« becomes negligible as compared with the »relative errors«. As to the interpretation of this statement it seems natural to think of the source of the »constant errors« as the residual effect of the non-specific chromogens of the urine after the colour correction.

For a quantitative discussion of the errors a more exact specification is required.

If we denote the true amount of 17-ketosteroids by ξ , our working hypothesis implies that one standard deviation, σ_a , is independent of ξ , while the other is proportional to ξ , say $\sigma_r \xi$. The total variation then has a standard deviation σ given by

$$(1) \quad \sigma^2 = \sigma_a^2 + \sigma_r^2 \xi^2.$$

As the observations with really small ξ 's are rather scarce, it did not seem worth while to spend much work on elaborate estimations of both σ_a and σ_r . A preliminary investigation,

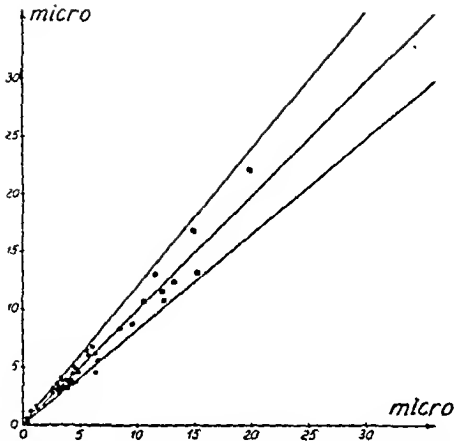


Fig. 4. Duplicate micro-benzene analyses.

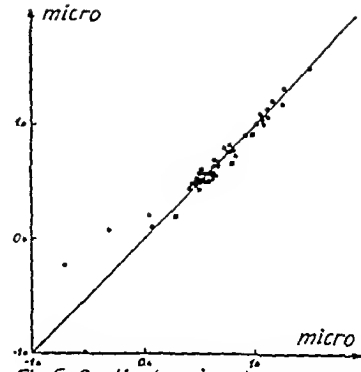


Fig. 5. Duplicate micro-benzene analyses. Logarithms.

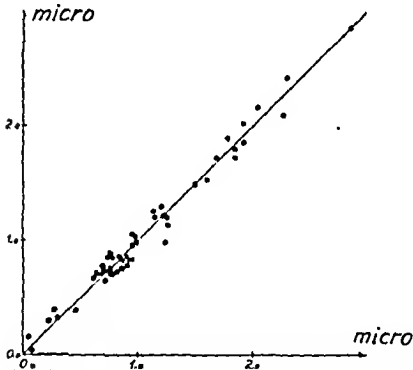


Fig. 6. Duplicate micro-benzene analyses. Transformed by $y = \text{arc sinh } x$.

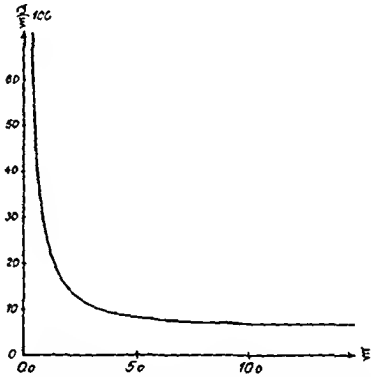


Fig. 7. Micro-benzene analyses. Coefficient of variation as a function of true amount of 17-ketosteroids.

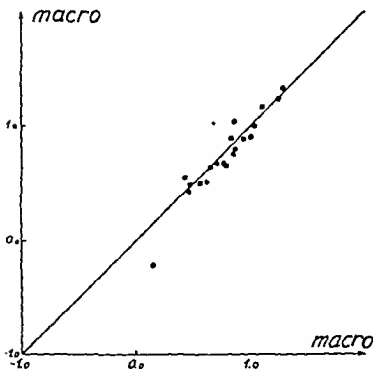


Fig. 8. Duplicate macro analyses. Logarithms.

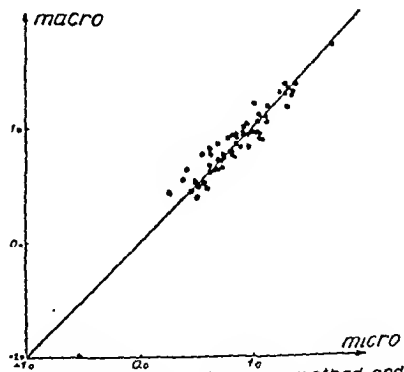


Fig. 9. Comparison of macro method and micro-benzene method. Logarithms.

involving some trial and error, leads to a rough estimate of $\frac{1}{4}$ for the ratio σ_r/σ_a . We then modified the hypothesis to

$$(2) \quad \sigma^2 = \sigma_a^2 \left(1 + \frac{\xi^2}{16}\right).$$

In order to control this hypothesis we noticed that when applying the transformation

$$(3) \quad y = \operatorname{arcsinh} \frac{x}{4} = \log \left(\frac{x}{4} + \sqrt{\frac{x^2}{16} + 1} \right)$$

to the observed amounts of 17-ketosteroids we should — according to the differential coefficient

$$(4) \quad \frac{dy}{dx} = \frac{1}{\sqrt{x^2 + 16}}$$

— get quantities with a constant standard deviation, viz.

$$(5) \quad \sigma_y = \frac{\sigma_a}{4}.$$

In Fig. 6 the effect of the transformation is demonstrated: Now the points are evenly distributed around the identity line for small as well as for large observations. The modified hypothesis (2) therefore seems acceptable and thus, according to (5), σ_a may be estimated from the transformed duplicate observations.

We find $\sigma_a \approx 0,258$ and consequently $\sigma_r \approx 0,061$. Inserting in (1) we may compute a table of the estimated σ for different ξ 's. Since the amounts of 17-ketosteroids most frequently met with in the clinical analyses exceed 2.5 mg./24 hrs., in which domain the second term on the right of (1) prevails it seems convenient to tabulate both σ and σ/ξ (Table 1). In Fig. 7 σ/ξ is shown as a function of ξ .

The accuracy of the standard routine method is investigated in 20 duplicate determinations. The logarithms of the amounts of 17-ketosteroids found are plotted in Fig. 8. Apart from one pair of observations they all lie within the range 2.5 mg./24 hrs. to 21.1 mg./24 hrs., and in this interval only the »constant relative error« makes itself felt. Estimating as usual the variance of the logarithms and transforming back to the original observations, we find a standard deviation of

13 per cent. On comparing with Table 1 we learn that for amounts of about 2.5 mg./24 hrs. the two methods are equivalent, but for larger amounts the micro-method is far more accurate. At about 10 mg./24 hrs. the standard deviation for the micro-method is only about half of the standard routine method.

Table 4.

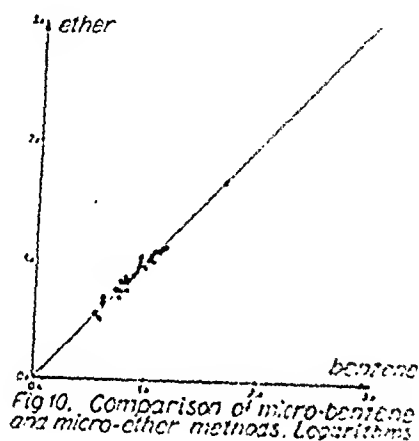
ξ mg./24 hrs.	σ mg./24 hrs.	σ/ξ per cent
0.5	0.260	52.0
1.0	0.266	26.6
2.5	0.304	12.1
5.0	0.412	8.2
10.0	0.694	6.9

The relation between the content of 17-ketosteroids and the standard deviation (micro-benzene-method).

In a series of 61 experiments we have compared the two methods directly in order to see whether the results deviate systematically from each other. The logarithms are plotted in Fig. 9; the diagram shows no obvious bias, and a comparison of the mean of the differences with its mean error confirmed the impression ($t = -1.20$). Again it should, however, be noticed that the experiments cover only the range 2.5 mg./24 hrs. to 34.4 mg./24 hrs. The question has therefore not been settled for small amounts of 17-ketosteroids. As to the variation of the differences we estimate the relative standard deviation at 15.7 per cent which seems rather large as compared with the standard deviation for each of the methods. The reason for

this may simply be that the estimate of the variance for the standard routine method, resting upon 19 degrees of freedom only, just happened to be fairly small. We may conclude that the true standard deviation for the standard routine method presumably is somewhat larger than the estimate found (13 per cent).

The comparison of the »micro-benzene-« and the »micro-



ether-method« is illustrated in Fig. 10, where the logarithms of the 17-ketosteroid values found are plotted against each other. The comparison covers the range 3.6 mg./24 hrs. to 46.8 mg./24 hrs. As the points lie evenly around the identity line, no systematic difference between the results of the two methods prevails, and only a »constant relative error« can be located. The mean square of the differences is found to be 0.00164 which does not deviate significantly from what we found for the difference between parallel determinations by the »micro-benzene-method«, viz. 0.00254.

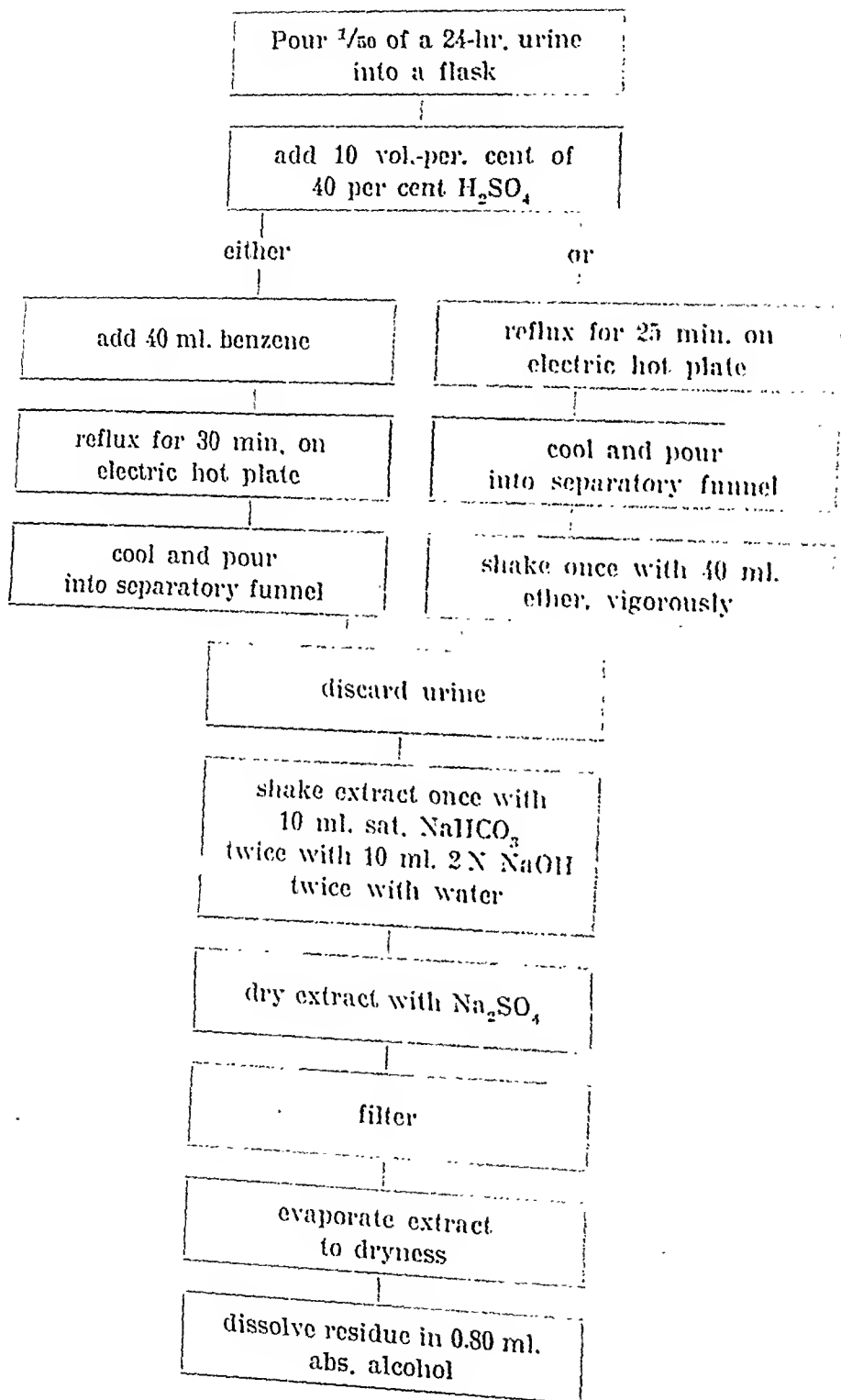
If the ether and benzene analyses had been carried out just as duplicate analyses, i. e. on different days, possibly with different batches of reagents and performed by different operators, the conclusion naturally would be that the two methods are equivalent in accuracy also. But the factors mentioned being identical, there is a considerable risk that the

variance estimated represents practically the errors in one single step of the procedure only, viz. the step where the two methods differ. If the presumption be correct, the errors in this step of the ether procedure should have a variance exceeding that of the errors in the corresponding step in the benzene method. Consequently, the total variance of the ether method should exceed the total variance of the benzene method. Now the presumption may be wrong or the estimate of the variance may happen to be fairly large. There is therefore a possibility that the two methods are equally accurate. In order to obtain a definite result a new series of experiments is required.

An analysis of the data underlying the range of 17-ketosteroids for normal persons given by *Hamburger* (1948 a, b) has shown that the logarithms may be taken to be normally distributed and that the variance independently of the age is about 0.0225. As the corresponding variance for the accuracy of the standard routine method was estimated at 0.0025, cf. the above mentioned standard deviation of 13 per cent, the technical errors are quite insignificant as compared with the biological variations. *Therefore the change of technique is of no consequence as regards the range of normality.*

SUMMARY

The purpose of this investigation has been to find the optimal conditions for hydrolysis and extraction of 17-ketosteroids in very small amounts of urine (1/50 of a 24-hour specimen). Simultaneous hydrolysis and benzene extraction and separate hydrolysis followed by ether extraction in separatory funnel were performed according to the technical description below:



By these »micro-methods« for extraction in combination with Callow's modification of the Zimmermann-reaction and the use of a colour correction nomogram the determination of the total neutral 17-ketosteroids in urine can be carried out in the course of two hours. The two methods give the same results as the standard routine method hitherto used in this laboratory (combined hydrolysis and benzene extraction of half the volume of a 24-hour urine, three 1½-hour refluxings on steam boilers). When carbon tetrachloride was substituted for benzene, the combined hydrolysis and extraction gave very poor recoveries, and by independent hydrolysis and extraction the recoveries were somewhat lower and more variable than by the benzene- or ether-method.

The advantages of the »micro-methods« are obvious: They are considerably less time-consuming than the ordinary methods; the amount of reagents required is minimized and the analyses can be performed regardless of special laboratory equipment, except of a photometer. As the »micro-methods« (at any rate the benzene method, but probably also the ether method) are even more accurate than the ordinary large-scale analysis, they are recommended as a means for determination of the total neutral 17-ketosteroids in the urine. The small amount of extract, however, does not permit the separation of the various components of the total neutral fraction.

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Announcements from the Endocrinological Societies

LIST OF PAPERS READ AT THE MEETING OF THE SCANDINAVIAN SOCIETIES FOR ENDOCRINOLOGY HELD IN STOCKHOLM ON SEPTEMBER 25—26, 1948

- Francis, T.* (Copenhagen): Experimental production of chromophobe pituitary adenomas.
- Hamburger, Chr. & Østergaard, E.* (Copenhagen): Quantitative determination of antigonadotrophic substances.
- Østergaard, E. & Hamburger, Chr.* (Copenhagen): Antigonadotrophic substances in women treated with pregnant mares' serum hormone.
- Jores, A.* (Hamburg, guest): Rythmus und innere Sekretion.
- Stahle, J.* (Uppsala): Diurnal rhythm of liver glycogen after hypophysectomy.
- Persson, B.* (Uppsala): Influence of castration, pregnancy and oestrin upon the lymphatic tissue of the spleen.
- Kinnunen, O.* (Helsinki): The effect of methylthiouracil and sulfonamide on the concentration of iodine and cholesterol in the rat.
- Sandblom, Ph.* (Stockholm): Ovarian pseudo-hermaphroditism. Demonstration of a patient 7 years old.
- Törnblom, N.* (Uppsala): On the hormonal regulation of calcium and phosphor in the blood.
- Liavaag, K.* (Oslo): Surgical treatment of hyperparathyroidism.
- Waldenström, J.* (Uppsala): Gynaecomasty as part of a definite syndrome.
- Frisk, R.* (Stockholm): Obesity and its treatment.
- Luft, R.* (Stockholm): The effect of desoxycorticosterone acetate and sodium chloride on the blood pressure.
- Fonss-Bech, P.* (Copenhagen): Investigations into enterogastrone.
- Madsen, V., Pedersen-Bjergaard, K., Roholt, K. & Tonnesen, M.* (Copenhagen): Antigen- and pyrogen content of commercial chorionic gonadotrophin preparations.
- Jacobsen, D.* (Lund): The influence of the corticotrophic hormone upon the mammary growth in rats.

- Pedersen-Bjergaard, K.* (Copenhagen): Oestrogenic and gonadotrophic substances in the urine during pregnancy.
- Jensen, C. C.* (Copenhagen — Malmö): Investigations into 17-ketosteroids.
- Nielsen, Aa. Theil* (Copenhagen): The excretion of phenolic 17-ketosteroids in pregnant mares.
- Hultqvist, G.* (Stockholm): Gigantism in youngs of rats with experimental diabetes.
- Lindberg, K.* (Stockholm): On a peculiar secretion picture accompanied by fuchsinophilia of the fetal adrenal cortex.
- Tonnesen, M. & Pedersen-Bjergaard, K.* (Copenhagen): On the protracted effect of oestradiol monobenzoate and of oestradiol dipropionate in subcutaneous oil deposits.
- Ingelman-Sundberg, A.* (Stockholm): The influence of E-avitaminosis upon the ovarian production of oestrogenic hormone.
- Walaas, O.* (Oslo): The metabolism of the uterine muscle.
- Johansson, H.* (Stockholm): The influence of the endometrial stroma upon the development of hormonally conditioned epithelial changes.
- Bruun, A. F., Hemmingsen, A. M. & Møller-Christensen, E.* (Copenhagen): On experimental ripening in the eel (*anguilla anguilla*).
- Carstam, S. Ph.* (Stockholm): The hormonal regulation of colour changes in crustacean.

DANISH SOCIETY FOR ENDOCRINOLOGY

11. Meeting, Oct. 28, 1948, State Serum Institute, Copenhagen.
- M. Sprechler:* A survey of the adrenal cortical hormones with special reference to their urinary excretion in normal and pathological cases.
- E. Rydberg:* Observations on the crystallization of the mucus from the cervix uteri.

